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ANALYTICAL STUDY OF ELECTROPHORETIC
CHARACTERIZATION OF KIDNEY CELLS

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1. SCOPE

This report documents two phases of electrokinetic studies performed during the period August 6, 1976-June 5, 1978. The first phase involved studies of embryonic kidney cells as a follow up to the MA-011 Electrophoresis Technology Experiment which was conducted during the Apollo-Soyuz Test Project (ASTP) in July 1975. The objectives of this experiment included: a. development of space-rated electrophoresis equipment for separation of biological cells; b. refinement of handling procedures for transporting living cells to and from space while maintaining cell viability and medium sterility; c. performance testing of the electrophoresis unit (EU) with a mixture of aldehyde-treated red blood cell populations with different electrophoretic mobilities; and d. application of the EU to the resolution of electrophoretic subpopulations from whole populations of human peripheral blood lymphocytes and embryonic kidney cells.

The results of the preliminary postflight analyses were published (1) but did not include complete analyses of the agreement between the predicted and observed behavior of all the samples. Preliminary theoretical analyses were reported (2) for the behavior of the red cell test samples and a computerized mathematical model was developed (3) and used to predict the red cell sample behavior. The objectives of the first study phase reported here were to complete the postflight analysis of the performance of the ASTP zone electrophoresis experiment involving embryonic kidney cells. Portions of this work have been published in a comprehensive report on the MA-011 experiment (12).

The second phase of the contracted studies was aimed at the production of standard particles for electrophoresis. This work was undertaken in response to a need for standardization of methods for producing, calibrating and storing electrophoretic particle standards which could be employed in performance tests of various types of electrophoresis equipment of interest to NASA. Promising procedures were tested for their suitability in the production of standard test particles from red blood cells and samples of particles were distributed to investigators at Marshall Space Flight Center and other NASA Contractors.

2. ANALYSIS OF THE MIGRATION BEHAVIOR OF KIDNEY CELLS IN THE ASTP MA-011 FLIGHT EXPERIMENT

2.1 Background

Human embryonic kidney cells were chosen as a candidate cell population for electrophoresis due to the interest in obtaining fractions of kidney cell populations which are avid producers of urokinase. Urokinase is an enzyme which is believed to operate in the normal control of blood clot propagation by activating plasminogen, a clot lysing enzyme (4). While urokinase is

recognized as a useful therapeutic agent in facilitating blood clot lysis in patients (5), its use has been limited by the excessive expense of producing human urokinase (6). Urokinase is produced by fetal human kidney cells in tissue culture, but it has been reported that only about 5-30% of the cells from the cortex of the kidney produce the enzyme (7). Thus it was estimated that significant increases in production efficiency would be achieved if the avid producers could be isolated from the whole kidney cell population. Ground-based studies indicated that urokinase-producers could be enriched by electrokinetic fractionation, but the separations were compromised by the rapid sedimentation of the cells and convective disturbances due to Joule heating.

Two samples of kidney cells were processed in the electrophoresis apparatus during the ASTP MA-011 flight experiment. The first run in column #3 proceeded as planned but the second run in column #7 was aborted due to a leak problem. Column #3 was returned to earth in the cryogenic freezer. At Marshall Space Flight Center, the frozen sample core was pushed out of the column into a teflon tube which was serially sectioned under sterile conditions to give approximately 5 mm length plug segments. These were bagged, stored at -176°C , and transported to Abbott Laboratories (N. Chicago, Ill.). There, the specimens were thawed rapidly at 37°C , centrifuged, counted for viable and nonviable cells and then planted for growth in tissue culture. Samples of the cultured cells which grew were tested for urokinase production while other samples were examined by analytical particle electrophoresis. Where sufficient sample materials were available, erythropoietin and human granulocyte conditioning factor (HGCF) production were also assayed.

During the period immediately following the ASTP flight experiment the above mentioned biological assays were conducted by Abbott Laboratories while this laboratory performed the electrophoretic analyses. While these data were encouraging, there were indications of unexpected behavior by the kidney cells in the flight experiment which prompted the following examination of the degree of agreement between the theoretically predicted and observed migration behavior and factors which might have operated to produce discrepancies.

A second feature of these studies was to collect electrokinetic data for kidney cell preparations in an alternate suspending medium which might be employed in future electrophoretic separation experiments. The design and operation constraints of the MA-011 experiment made it necessary to employ one electrophoresis buffer (A-1) for all processed samples (red cells, lymphocytes and kidney cells). Abbott Laboratories pointed out that this buffer represented a compromise medium and that in future missions their alternate buffer which was optimal for kidney cell maintenance should be considered.

2.2 Mathematical Expressions

2.2.1 Location of sample slices in flight column.

The longitudinal position of each sample slice in the flight column was computed from the mass of the sample slice and the dimensions of the flight column. The volume of each sample slice, $V(\text{cc})$, was obtained from the sample mass, $m(\text{g})$, and the density, $\rho(\text{g/cc})$, of A-1 buffer:

$$V = \frac{m}{\rho} \quad [1]$$

The density of A-1 buffer at 11°C is 1.025 g/cc (). The length of flight column, (mm), occupied by the sample slice was then computed assuming uniform cylindrical bore of the flight column:

$$l = \frac{V}{\pi R^2} = \frac{V}{\pi (3.175 \text{ mm})^2} \quad [2]$$

where R is the radius of the flight column in mm, and V is expressed in mm^3 .

2.2.2 Basic electrophoretic mobility relationships.

A charged particle when exposed to a voltage gradient will migrate toward the electrode of opposite charge. The electrophoretic mobility of the particle, U_e , is defined as:

$$U_e = \frac{V_e}{E} \quad [3]$$

where U_e = electrophoretic mobility in $\mu\text{m/sec/volt/cm}$
 V_e = electrophoretic velocity in $\mu\text{m/sec}$
 E = voltage gradient in volts/cm

In this treatment anodic mobility will be positive, i.e., a negatively charged particle which migrates to the anode would have a positive mobility.

When a suspension of charged particles is contained in a closed cylindrical tube whose walls carry a static charge, an applied voltage causes the particles to migrate and will produce circulation of the suspension, or electroosmosis. The observed velocity, V_o , of the charged particle will be the sum of the electrophoretic velocity, V_e , and the fluid velocity, V_w :

$$V_o = V_e + V_w \quad \text{in } \mu\text{m/sec} \quad [4]$$

The magnitude of V_w may be expressed as a function of the radial distance from the axis of the tube and of the fluid velocity at the tube wall:

$$V_w = V_s \left[\frac{2r^2}{R^2} - 1 \right] \quad [5]$$

where V_s = fluid velocity at the tube wall in $\mu\text{m}/\text{sec}$
 r = radial distance from the tube axis in cm
 R = radius of the tube in cm

The magnitude of V_s is a function of the charge on the tube wall and is proportional to the applied voltage. Thus an electroosmotic mobility, or osmobility, U_{osm} , is defined as:

$$U_{\text{osm}} = \frac{V_s}{E} \quad [6]$$

where U_{osm} = osmobility in $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$.

A negative tube wall charge produces a flow of the adjacent fluid toward the cathode, hence a negative osmobility expressed in the same sign convention adopted above for U_e .

The distance, $d(\mu\text{m})$, traversed by a particle in time, $t(\text{sec})$, at a point r cm from the tube axis will be:

$$d_r = V_o t = V_e t + V_s t \left[\frac{2r^2}{R^2} - 1 \right] \quad [7]$$

or in mobility terms,

$$d_r = Et \left[U_e + U_{\text{osm}} \left(\frac{2r^2}{R^2} - 1 \right) \right] \quad [8]$$

From equation [6] it is seen that at $r = 207R$ is an envelope, termed the stationary level, where the contribution of U_{osm} vanishes.

If a sample population of electrophoretically monodisperse particles is introduced into the chamber as a disc one particle in thickness and of radius r_p (cm), the final displacement and profile of the sample particles as a function of radial distance may be estimated with equation [6] if certain assumptions apply which will be outlined subsequently.

2.2.3 Electrokinetic relationships for the flight experiment.

Modifications of equation [8] must be made before it can be used to examine the correlation between the results of ground-based analytical

studies and the flight experiment since: a. the flight and ground experiments were conducted at different temperatures, and b. the effective voltage gradient, E , in the flight experiment was not monitored.

The predominant effects of temperature in the range from $\sim 10^\circ\text{C}$ to 40°C are due to alterations in the viscosity and the conductivity of the suspending medium. The relationship between electrophoretic mobility and zeta potential, ζ , (the electrostatic potential at the slip plane between the particle surface and the suspending medium) is accurately described for nonconducting biological cells by the Helmholtz-Smoluchowski equation:

$$U_e = \frac{\zeta D}{4\pi\eta} \quad [9]$$

where η and D are the viscosity in poise and the dielectric constant within the electrical double layer, which are assumed to be the same as the bulk values for the suspending medium. The validity of the assumptions are discussed in reference (8). At low zeta potentials (≤ 25 practical mV) the zeta potential may be expressed in terms of the surface charge density, σ , of the particle:

$$\zeta = \frac{4\pi\sigma}{\kappa D} \quad [10]$$

in which σ is the surface charge density in statcoulombs cm^{-2} and κ is the Debye-Hückel reciprocal length parameter:

$$\kappa = \left[\left(\frac{4\pi e^2}{DkT} \right) \sum_i n_i z_i^2 \right]^{1/2} \quad [11]$$

in which e is the charge on the electron, k is the Boltzman constant, T is the absolute temperature, and D is the dielectric constant of the medium which contains the number, n_i , ions per unit volume of type i with a valency of charge z_i .

Substitution of [10] into [9] gives:

$$U_e = \frac{\sigma}{\eta\kappa} \quad [12]$$

At two different temperatures, e.g. 10°C and 40°C , the ratio of particle mobilities in a single suspending medium will be:

$$\frac{U_e^{10}}{U_e^{40}} = \frac{\sigma^{10}/\eta^{10}\kappa^{10}}{\sigma^{40}/\eta^{40}\kappa^{40}} \quad [13]$$

and substitution of [11] into [13] gives:

$$\frac{U_e^{10}}{U_e^{40}} = \frac{\sigma^{10} \eta^{40} (D^{40} T^{40})^{1/2}}{\sigma^{40} \eta^{10} (D^{10} T^{10})^{1/2}} \quad [14]$$

For simple ionic media whose dielectric properties are similar to those of water the $(DT)^{1/2}$ ratio is nearly 1. With the values for water the ratio is:

$$\left[\frac{D^{40} T^{40}}{D^{10} T^{10}} \right]^{1/2} = \left[\frac{73.15 \times 313.2}{83.83 \times 283.2} \right]^{1/2} = 0.982$$

In the temperature range from 10 to 40°C, the correction for an interval of 15°C is less than one percent, so that this correction is of little consequence. Variations in surface charge density can also be assumed to be experimentally insignificant based on the observations of Mehrishi and Seaman (9) that corrections for viscosity changes in the temperature range 1°-40°C gave constant viscosity corrected mobility values for human red cells in phosphate buffered saline and saline with ionic strengths of ~ 0.15 M and for quartz particles in phosphate buffered saline.

Thus equation [14] may be simplified and rearranged to give:

$$U_e^T = \frac{U_e^{25} \eta^{T1}}{\eta^{T2}} \quad [15]$$

Similar considerations apply to U_{osm} so that substitution of [15] into [8] gives the particle migration distance, d_r^T , for temperature, T, in terms of mobility values obtained at a standard temperature of 25°C:

$$d_r^T = \left[\frac{\eta^{25}}{\eta^T} \right] \left[U_e^{25} + U_{osm}^{25} \left(\frac{2r^2}{R^2} - 1 \right) \right] E^T t \quad [16]$$

The final problem is the definition of the voltage gradient, E^T , at the operating temperature, T. By definition the voltage gradient is the voltage difference, V^T , in volts through a length, ℓ , in cm of electrophoresis column:

$$E^T = \frac{V^T}{\ell} \quad [17]$$

Since the electrophoresis columns were operated at constant current, I, the voltage gradient can be calculated according to Ohms law from the resistance, R^T , at temperature T:

$$E^T = \frac{IR^T}{\ell} \quad [18]$$

The resistance for a uniform bore tube can be expressed in terms of the specific conductance or conductivity, κ , of the contained electrolyte and the cross sectional area, a , of the tube:

$$R^T = \frac{\ell}{\kappa^T a} = \frac{\ell}{\kappa^T \pi R^2} \quad [19]$$

where R is the radius of the tube. Substitution of [19] into [18] gives:

$$E^T = \frac{I}{\pi \kappa^T R^2} \quad [20]$$

Incorporation of [20] into [16] gives an equation describing the migration distance in terms of the conditions of the flight experiment and ground-based analytical electrokinetic data. The last issue is to establish the reference point for the migration distance measurements. The zero point for measurement of cell migration in the flight columns is the end of the column which rests against the sample slide. Where the sample slide has a thickness, $d_o = 3.18$ mm, a particle at the midpoint of the sample plug must migrate $d_o/2 = 1.59$ mm to reach the column zero point. Thus the final band migration expression provides the distance, d_r^T (mm), traversed into the column at temperature, T , by the particles originally located at the longitudinal midpoint of the sample plug and at a distance, r (cm), from the tube axis:

$$d_r^T = \left[\frac{\eta^{25} I \times 6 \times 10^{-2}}{\pi R^2 \kappa^T \eta^T} \right] \left[U_e^{25} + U_{osm}^{25} \left(\frac{2r^2}{R^2} - 1 \right) \right] t - \frac{d_o}{2} \quad [21]$$

where η^{25} and η^T are the viscosities in poises of A-1 buffer at 25°C and at the run temperature, T , respectively; I is the constant current setting in amperes for operation of the flight column; R is the radius of the electrophoresis column in cm; κ^T is the conductivity in mho/cm of A-1 buffer at temperature, T ; U_e^{25} is the mean electrophoretic mobility (anodic) in $\mu\text{m/sec/volt/cm}$ of the particles at 25°C in A-1 buffer; U_{osm}^{25} is the electroosmotic mobility (anodic) of A-1 buffer in flight type columns at 25°C; t is the electrophoresis run time in minutes; d_o is the sample plug thickness in mm; and 6×10^{-2} is a combined factor for converting μm to mm and minutes to seconds.

The following assumptions are made in applying equation [21] to the prediction of the migration behavior of samples in the zone electrophoresis experiment:

- a. Applied current, I is constant.
- b. No radial or longitudinal thermal gradients are developed during column operation.
- c. There are no hydrodynamic or electrostatic interactions between individual particles in the sample.
- d. There is no aggregation of sample cells with differing mobilities.
- e. The suspending medium is Newtonian and exhibits laminar flow (no convective disturbances).
- f. The electrophoresis column is a closed system with no net fluid transport through the column.
- g. Sample cells are nonconducting and electrokinetically stable for the duration of the run and their mobilities are unaltered by freeze-thaw procedures.
- h. The cells behave as the sum of behaviors of the individual particles which remain at a fixed radial distance throughout the run.
- i. Compression of the electrical field through the sample plug aperture does not significantly alter the magnitude of final migration distance.
- j. Current density is radially uniform in the column.

2.3 Parameter Values for Flight Experiment

The values of critical variables in the theoretical expressions in the preceding sections were as follows:

Electrophoresis column length = ℓ = 14.94 cm
 Electrophoresis column radius = R = 0.3175 cm
 Sample plug radius = 0.239 cm
 Sample plug thickness = d_0 = 0.312 mm
 Current setting = I = 4.0×10^{-3} amp
 Run time = t = 75 min. (kidney cells, column 3)
 Average run temp. = T = 11°C (kidney cells, column 3)

Physical properties of A-1 buffer (2):

Temp. (°C)	κ^T (mho/cm)	η^T (poise)	ρ^T (g/cc)
10	6.7×10^{-4}	16.7×10^{-3}	1.0253
15	7.6×10^{-4}	-	-
18	-	13.3×10^{-3}	1.0237
20	8.6×10^{-4}	-	-
25	9.6×10^{-4}	11.1×10^{-3}	1.0223
30	10.6×10^{-4}	9.87×10^{-3}	1.0213
35	11.9×10^{-4}	8.87×10^{-3}	1.0203

2.4 Experimental Methods

2.4.1 Analytical particle electrophoresis

Analytical particle electrophoresis was carried out as described by Seaman (8,10) with 2 mm i.d. cylindrical chambers equipped with either reversible Ag/AgCl electrodes or irreversible Pt electrodes. All measurements were obtained with dilute cell suspensions ($\leq 0.1\%$ by volume). Chamber temperatures were maintained at 25.0°C by a circulating constant temperature bath. The accuracy of the chamber calibrations were checked with human red blood cells as a standard particle at the beginning and end of each series of measurements (8). Field strengths did not exceed 5 volts/cm and amperages through the chambers were typically < 2.5 mA for physiological saline and < 1 mA for low ionic strength buffers such as A-1 buffer. The polarity of the electrodes was reversed after each particle velocity measurement to minimize adverse reactions at the electrodes (8,10). Details of the harvesting and washing procedures used for kidney cells will be given under "Results". Electrophoretic mobility values are reported as anodic mobilities, i.e. a negatively charged particle such as a biological cell will have a positive mobility value.

2.4.2 Embryonic kidney cells

All human embryonic kidney cells were supplied by the Molecular Biology Research Division of Abbott Laboratories (North Chicago, Illinois). The cells were typically grown to confluency in Eagles medium (E-199) containing 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 in air (6). Cells were grown in plastic tissue culture flasks (Falcon) with 75 cm^2 growth surface unless otherwise noted. After approximately 7-10 days when the cells reach confluency, the growth medium was replaced by maintenance medium (E-199 + 1 or 2% fetal calf serum) in order to minimize overgrowth and "pilling" during shipment to our laboratory. Deviations from these procedures and variations in cell harvesting procedures during the ground-based studies will be documented under "Results".

The kidney cells for the flight samples had been prepared at Abbott Laboratories. Cells from primary cell lot number 915 were grown to confluency in growth medium in 75 cm^2 Falcon flasks. The cells were harvested as follows. The growth medium was decanted from each flask. The flask was rinsed once with phosphate-buffered saline (PBS) (as described under "Methods") and the PBS was decanted. In order to release the cells from the flask surface 3 ml of trypsin-PBS (0.25% w/v Microbiological Associates trypsin giving 450 Units trypsin/ml in PBS) was added and the flask was incubated at 37°C until $\sim 90\%$ of the cells were released (15-30 min). Then 7 ml of E-199 growth medium was added and the suspension was aspirated 15-20 times with a 10 ml pipette and rubber bulb. The suspensions from several flasks were pooled and centrifuged at $\sim 200\text{ xg}$ at room temperature for approximately 10 minutes followed by two to three washes in A-1 buffer at 37°C and then suspension in A-1 buffer. Aliquots of the sample suspension were then rate-frozen in flight sample keys. The whole procedure required < 3 hours.

2.4.3 Media

The following media were frequently employed for the indicated purposes. Water double distilled in pyrex ware and analytical grade reagents were used in this preparation.

Phosphate buffered saline (PBS) for washing kidney cells during harvest procedures and for a limited number of mobility measurements had the following composition:

<u>Substance</u>	<u>g/liter</u>	<u>Millimolarity</u>
NaCl	8.50	145.
Na ₂ HPO ₄ ·7H ₂ O	2.07	7.72
NaH ₂ PO ₄ ·H ₂ O	0.315	2.30

The osmolality was 288 milliosmoles/Kg based on freezing point depression and the pH was 7.2 at 25°C.

The ASTP flight experiment buffer, A-1, had the following composition:

<u>Substance</u>	<u>g/liter</u>	<u>Millimolarity</u>
Na ₂ HPO ₄ ·7H ₂ O	0.472	1.76
KH ₂ PO ₄	0.050	0.367
NaCl	0.375	6.42
Na ₂ EDTA·2H ₂ O	0.125	0.336
D-glucose	40.0	222.
Glycerol	47.3	514.

The calculated ionic strength is 9.7×10^{-3} mole/l. The osmolality in the absence of glycerol is 248 mOsm/Kg while that in the presence of glycerol is 840 mOsm/Kg. At 25°C the pH is 7.28 ± 0.03 , density, ρ , is 1.022 g/cc, dynamic viscosity, η , is 1.11×10^{-2} poise, and the conductivity, κ , is 9.6×10^{-4} mho/cm (2).

"Standard" or "physiological" saline used in the context of these studies refers to 0.15 M NaCl (8.77 g NaCl/l) whose pH has been adjusted to 7.2 ± 0.2 with 0.15 M NaHCO₃ (12.6 g NaHCO₃/l). This medium is both the wash and reference electrophoresis medium for red cells in the calibration of the analytical particle electrophoresis equipment.

2.5 Results

2.5.1 Preflight electrokinetic data

During August and September, 1974, analytical electrophoretic mobility data were collected for kidney cells suspended in A-1 buffer. Two batches (995-1, 995-2) of kidney cells in growth medium representing two successive passages through tissue culturing were shipped by Abbott Laboratories approximately two days prior to confluency. Shipping time was less than

one day and upon receipt the cells were incubated for 1 day at 37°C prior to harvesting for electrophoretic studies.

The harvesting procedure consisted of removing the bulk of the culture medium, removal of the flask side opposite the cell layer with a hot knife, detaching the cell layer from the flask surface with a rubber policeman and suspension of the cells in the growth medium by repeated passages through a 9" Pasteur pipet. The cells were centrifuged from suspension at 600-700 xg and were resuspended and washed four times with ~ 100 volumes of phosphate-buffered saline pH 7.2 followed by one wash in A-1 buffer. All operations were carried out at room temperature (~ 21-23°C).

The electrophoresis experiment consisted of two parts: one to determine the distribution of electrophoretic mobilities of the kidney cells in A-1 buffer; and a second to determine whether any changes in electrophoretic mobility arise due to short term exposure of the kidney cells to the A-1 buffer. For lot 995-1, the electrophoretic mobilities of 40 cells in phosphate-buffered saline averaged 0.91 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ with a standard deviation of 0.05. Following transfer to the A-1 buffer, a series of 200 measurements were obtained and then after 4-1/2 hours the cells were transferred back into phosphate-buffered saline. Examination of 40 cells gave an average mobility of $0.92 \pm 0.06 \mu\text{m}/\text{sec}/\text{volt}/\text{cm}$. Thus no significant changes in electrophoretic behavior were produced by short term exposure to the low ionic strength A-1 buffer at 22-24°C.

The results of the 200 mobility measurements of the 995-1 kidney cells in the A-1 buffer are shown in Figure 1A. The observed range of mobilities was 1.32 to 2.12 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ with an average of 1.66 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ and a standard deviation of 0.16.

The second batch (995-2) of kidney cells was examined to determine whether reculturing cells from a primary culture altered the electrokinetic properties of the cell population. The electrophoretic mobility histogram obtained for these cells in A-1 buffer (Figure 1B) did not differ significantly from that of the cells from the first culture (Figure 1A). The mobility range was from 1.34 to 2.12 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ with a mean of 1.67 and a standard deviation of 0.19 for 95 cells.

These data showed that a. the whole kidney cell population displayed a broad range of electrophoretic mobilities in A-1 buffer so that subpopulations should be resolved by preparative electrophoresis; b. short term exposure of the cells to A-1 buffer did not produce alterations in their averaged electrokinetic properties; and c. two successive cultures of cells from a primary cell line displayed no significant differences in their electrophoretic mobilities in A-1 buffer.

Experiments were not conducted on kidney cells from different donors so that it was not established at that time whether cell lines from different donors displayed the same properties.

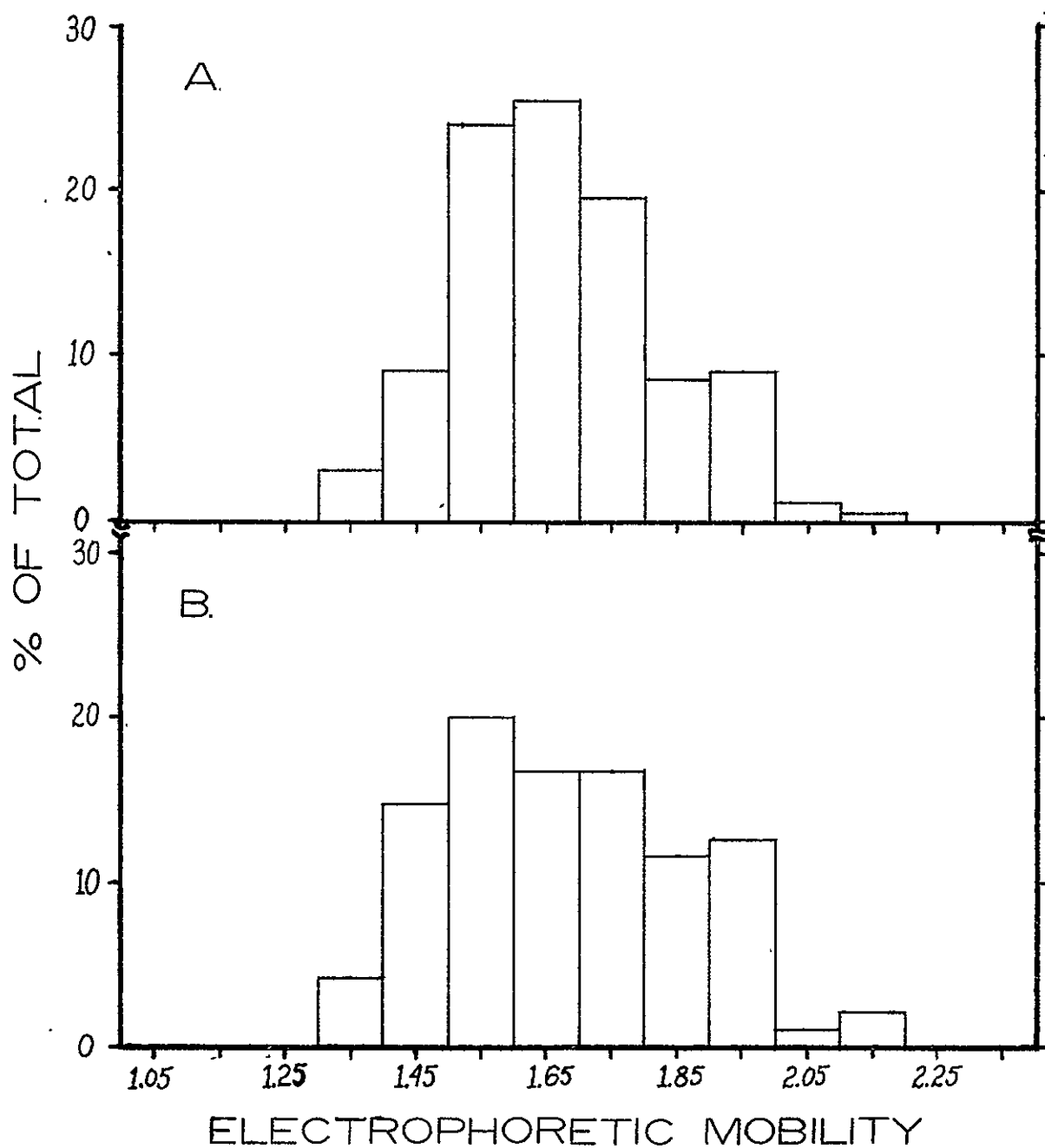


FIGURE 1. Electrophoretic Mobility Histograms for Successive Cultures of Kidney Cells. The percent of the total cell population in each interval is plotted as a function of the electrophoretic mobility in $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ of the cells in A-1 buffer at 25°C : A. Cell lot 995-1, first passage through culture, no. of cells examined = 200; B. Cell lot 995-2, second passage, no. cells = 95.

2.5.2 Migration behavior of kidney cells in the flight experiment

The flight samples of embryonic kidney cells each contained a total of approximately 2×10^6 cells. The sample in column 3 was electrophoresed for 75 minutes at an average column temperature of 11°C for the midpoint of the electrophoresis column. During the last 23 minutes of the run photographic difficulties precluded collection of column temperature data.

The returned frozen column was cut into 28 sample sections. The pH's of the sample segments ranged from 7.0 to 7.9. No bacterial contamination was observed.

The distribution of viable cells (based on dye exclusion) in the flight column sample slices has been reported (1). In addition to the viable cell counts, data was collected on the number of nonviable cells in all of the sample slices except numbers 1-3 and 13-14. The cell count data and the masses of the individual slices were used in the estimation of the original locations of each slice in the flight column with equations [1] and [2]. These data are given in Table 1 and are plotted in Figure 2 where the expected mobility, U_{e}^{25} , of the cells is plotted as a function of migration distance. The latter was computed with equation [21] as a first approximation with the assumption that the migration was not influenced by electroosmotic flow, i.e. $U_{\text{osm}}^{25} = 0$.

Based on the bore dimensions of the flight column the theoretical total sample volume is 4.73 cc. Summation of the computed sample slice volumes gives a total of 4.50 cc, or 95% of the theoretical. The calculated length of the column occupied by this volume is 14.2 cm (total column length was 14.9 cm).

About 84% of the original cell load (2×10^6 cells) were recovered. However, as noted above nonviable cell counts were not collected for five of the samples. The total recovery value of 1.9×10^6 cells listed in Table 1 involved the assumption that samples 13 and 14 each contained at least as many nonviable cells as did sample 17 which had the highest value (1.3×10^5) of all the counted samples. No nonviable cell counts were collected for 13 or 14 since the cells were "too numerous to count". No such assumptions were made for sample 1-3. Each was listed as containing no nonviable cells since neither were the counts collected nor were any qualitative statements made about the relative numbers of viable and nonviable cells. Employing the value of 1.9×10^6 cells as a reasonable estimate of cell recovery, about 40% of these cells were viable and 60% were nonviable.

All samples containing viable cells were cultured, and while normal adherence to the tissue culture flasks was observed in most cases, only cells from fractions 11,13,14,15,16,17 and 19 proliferated.

TABLE 1. KIDNEY CELL SAMPLE SLICE DATA FOR FLIGHT COLUMN 3

Slice No.	Mass (g)	Volume ^a (cc)	ℓ (mm) ^a	$\Sigma \ell$ ^a (mm)	Recovered Cells x 10 ⁻⁴			% ^d	Σ % ^d	pH
					Viable	NonViable	Total			
L	0.27805	0.2713	8.56	8.56	2.55	1.95	4.50	2.3	2.3	7.0
1	0.15400	0.1502	4.74	13.3	8.40	ND ^b	>8.40	4.4	6.7	7.8
2	0.15110	0.1474	4.65	18.0	6.60	ND	>6.60	3.4	10.1	7.9
3	0.14195	0.1385	4.37	22.3	1.40	ND	>1.40	0.7	10.8	7.8
4	0.16695	0.1629	5.14	27.5	3.90	0.90	4.80	2.5	13.3	7.8
5	0.16320	0.1592	5.03	32.5	1.95	0.90	2.85	1.5	14.8	7.8
6	0.15825	0.1544	4.88	37.4	1.05	0.75	1.80	0.9	15.7	7.6
7	0.14845	0.1448	4.57	42.0	1.45	0.90	2.35	1.2	16.9	7.8
8	0.16250	0.1585	5.01	47.0	2.10	3.30	5.40	2.8	19.7	7.8
9	0.17195	0.1678	5.30	52.2	3.75	2.10	5.85	3.0	22.8	7.8
10	0.15805	0.1542	4.87	57.1	3.45	2.40	5.85	3.0	25.8	7.8
11	0.15435	0.1506	4.75	61.9	1.95	3.15	5.10	2.6	28.4	-
12	0.17765	0.1733	5.47	67.4	2.40	4.50	6.90	3.6	32.0	7.6
13	0.15620	0.1524	4.81	72.2	4.80	(13) ^c	(17.8)	9.2	41.2	7.6
14	0.10100	0.0985	3.11	75.3	5.70	(13) ^c	(18.7)	9.7	50.9	7.4
15	0.13340	0.1301	4.11	79.4	1.64	8.70	10.3	5.3	56.3	7.5

TABLE 1. (Continued)

Slice No.	Mass (g)	Volume ^a (cc)	ℓ (mm) ^a	$\Sigma \ell$ ^a (mm)	Recovered Cells x 10 ⁻⁴			% ^d	Σ % ^d	pH
					Viable	NonViable	Total			
16	0.13925	0.1359	4.29	83.7	5.10	9.90	15.0	7.8	64.0	7.6
17	0.20405	0.1991	6.29	90.0	5.25	13.0	18.2	9.4	73.5	7.2
18	0.14975	0.1461	4.61	94.6	0.25	3.75	4.00	2.1	75.5	-
19	0.12015	0.1172	3.70	98.3	1.80	10.0	11.8	6.1	81.7	7.4
20	0.14915	0.1455	4.59	103	0	0.90	0.90	0.5	82.1	-
21	0.16115	0.1572	4.96	108	4.80	9.45	14.2	7.4	89.5	-
22	0.16695	0.1629	5.14	113	3.75	4.20	7.95	4.1	93.6	7.4
23	0.16230	0.1583	5.00	118	0.15	1.35	1.50	0.8	94.4	-
24	0.15635	0.1525	4.82	123	1.05	0.30	1.35	0.7	95.1	-
25	0.18545	0.1809	5.71	128	1.80	1.80	3.60	1.9	96.9	-
26	0.11970	0.1668	3.69	132	3.45	2.55	6.00	3.1	100	7.4
R	0.3231	0.3152	9.95	142	0	0	0	0	0	7.2
Σ		4.50	142		80	113	193			

^a Sample slice volume calculated with equation [1] and length of electrophoresis column, ℓ , occupied by slice calculated from equation [2]. $\Sigma \ell$ is the boundary of the sample slice distal to the origin of migration.

^b ND = not determined.

^c Values listed represent minimum estimates. Actual values not determined but were listed as "too numerous to count."

^d % is the calculated percentage recovery of viable and nonviable cells in each slice. The estimates in footnote c were included.

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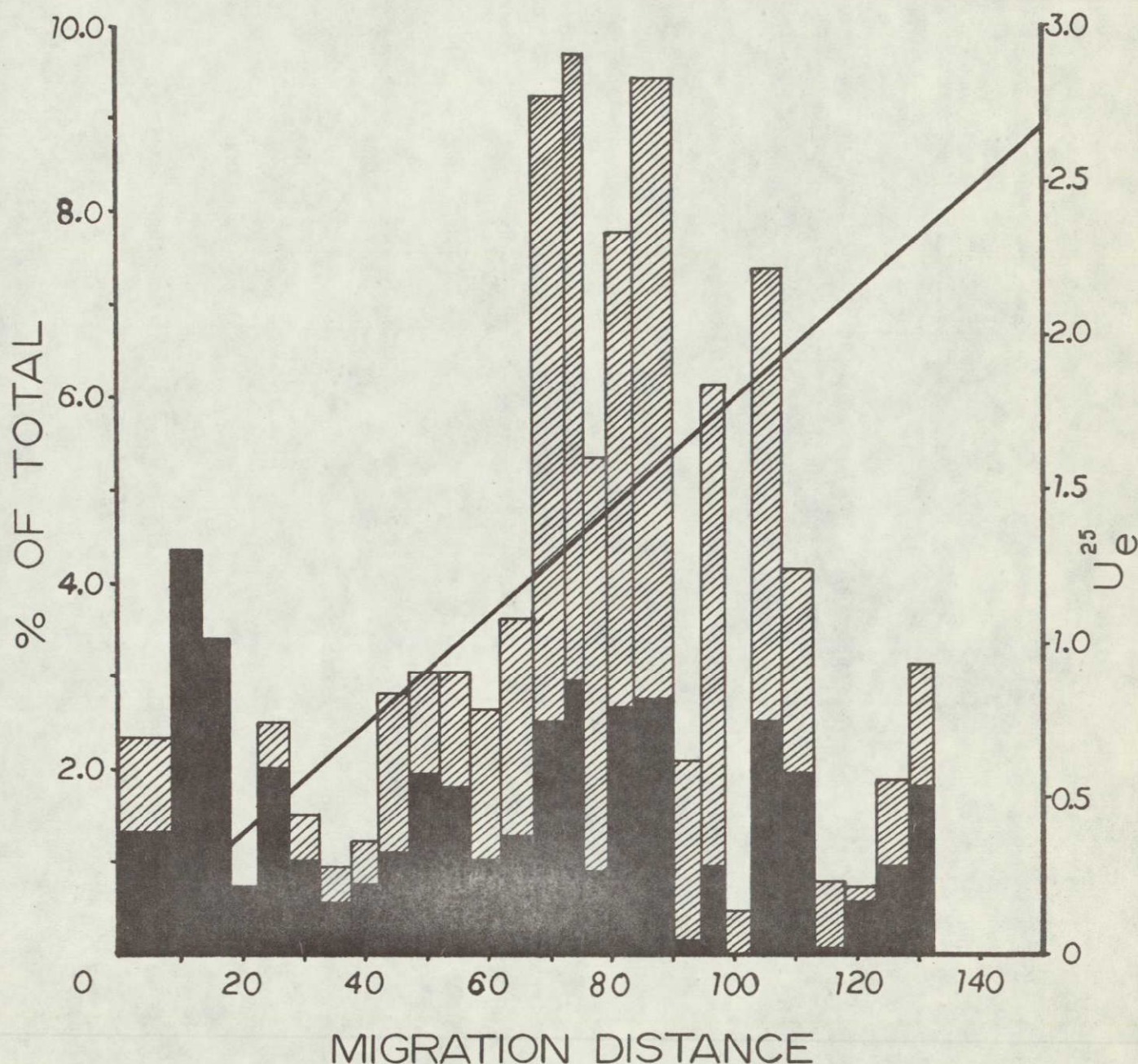


FIGURE 2. Migration Behavior of Kidney Cells During the Flight Experiment. Each bar in the graph corresponds to a single sample slice and gives the percentage of the total recovered cells in each and the computed migration distance in mm from the sample slide. The solid portion of each bar represents cells which were "viable" based on dye exclusion and the lined portion represents nonviable cells. The straight line plot gives the expected electrophoretic mobility ($\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$) of the cells at 25°C in A-1 buffer as a function of migration distance as calculated from equation [21] assuming no electroosmotic flow.

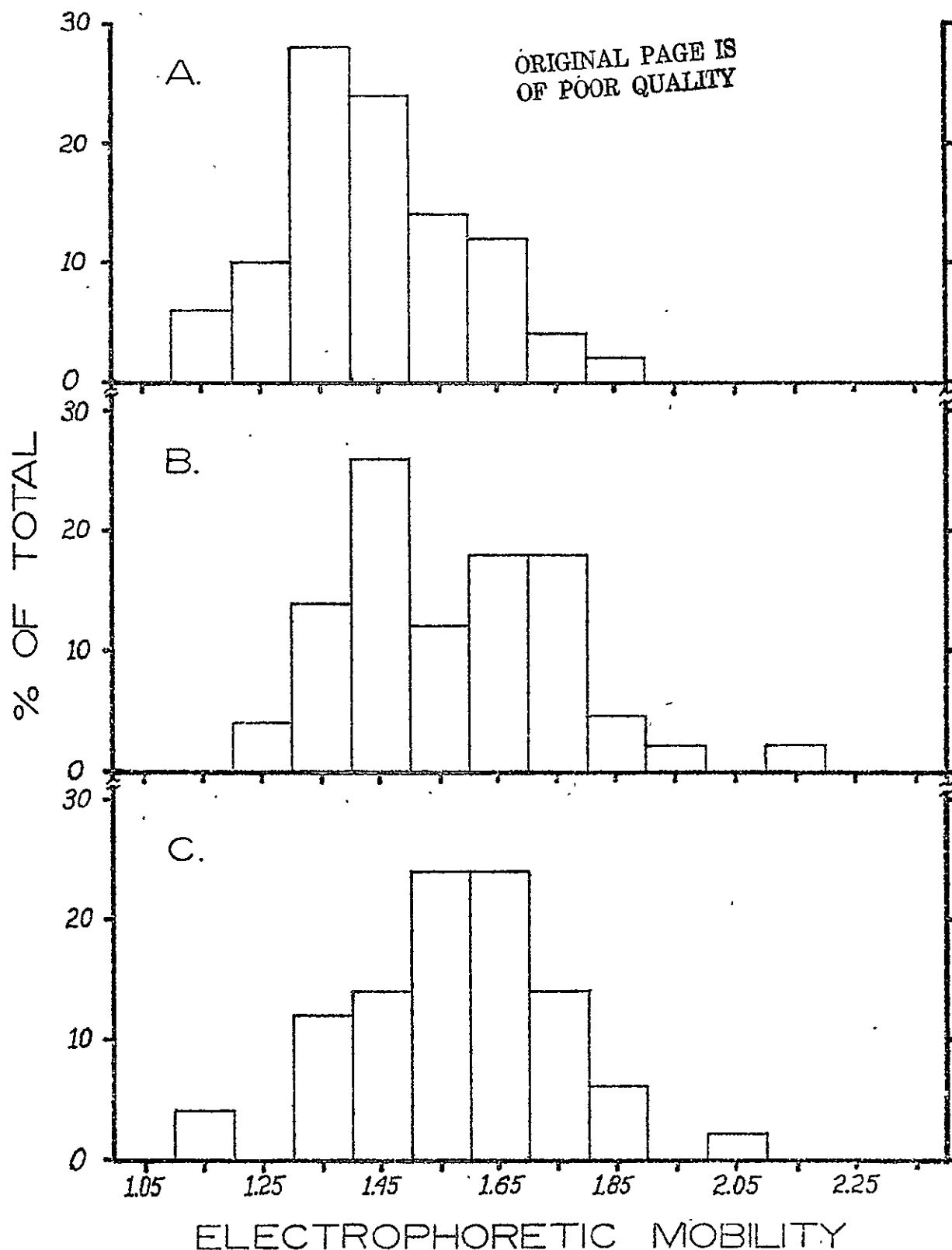


FIGURE 3. Electrophoretic Mobility Distributions for Kidney Cells Grown from Flight Sample Slices. The percentage of the total cells examined for each slice is given for each electrophoretic mobility interval ($\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$). Fifty cells were measured in A-1 buffer at 25°C for the second culture of kidney cells from the flight sample slices: A. slice 14; B. slice 17; and C. slice 19.

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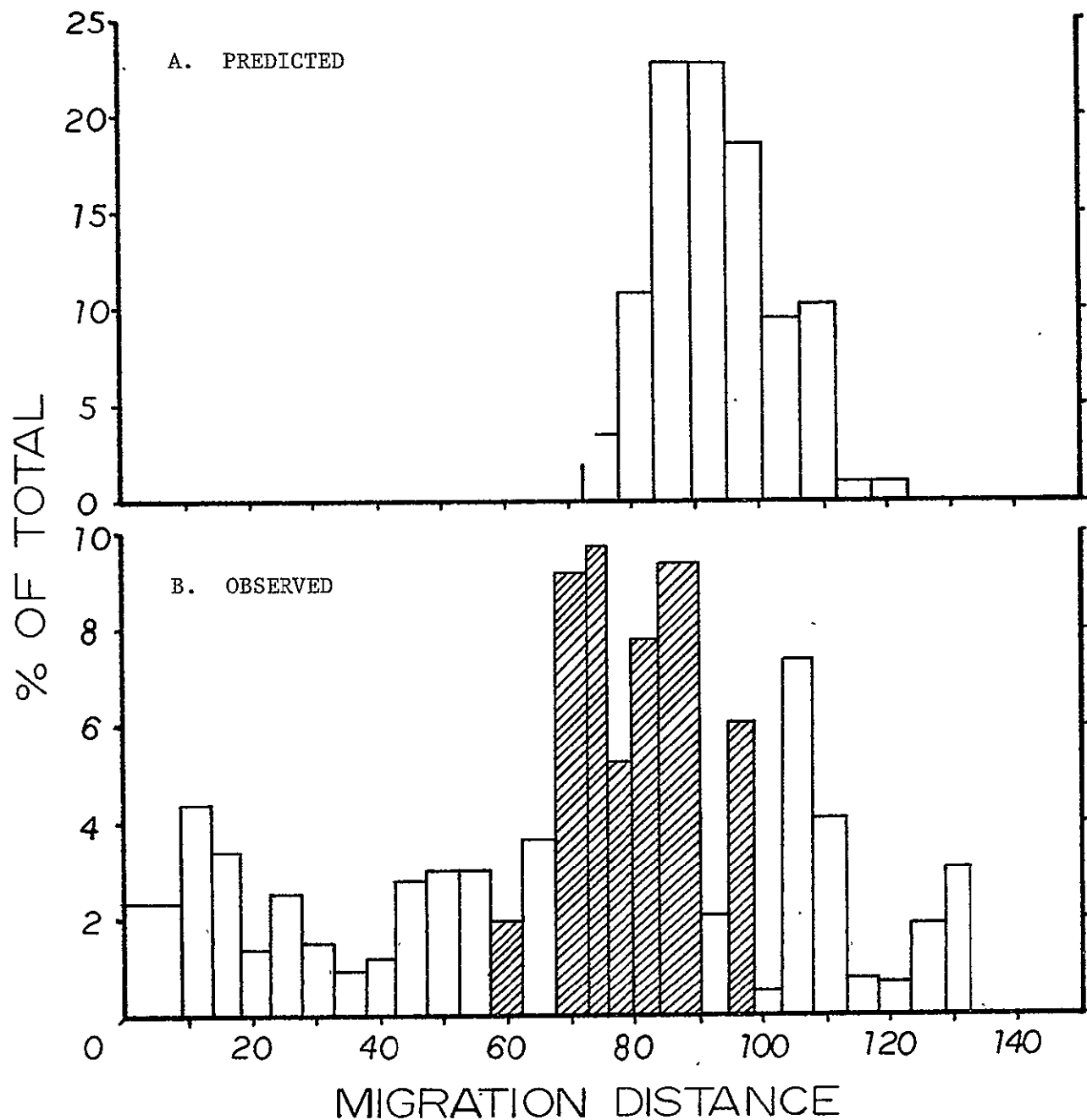


FIGURE 4. Comparison of Predicted and Observed Migration Behavior of Kidney Cells in the Flight Experiment. The bar graph gives the % of the total cell population located at different migration distances in mm in the flight column: A. Predicted behavior as calculated from equation [21] and the pooled preflight data in Figure 1 assuming $U_{Osm}^{25} = 0$; B. The observed migration of the recovered kidney cells in column 3 based on the data in Figure 2 and Table 1. The shaded bars represent the cell samples which grew in tissue culture. Note that different scales have been used for the ordinates in A and B.

column (Fig. 4B) about 50-60% of the recovered cells were located in this region, while about 5% were recovered at > 125 mm, and approximately 30% were distributed rather evenly through the 0-70 mm region.

Also indicated in Figure 4 are the sample slices which contained cells which grew in tissue culture. It may be significant that the only responsive cells were obtained from the region of the column which was predicted to contain the cells.

2.6.2 Comparison of predicted and observed electrophoretic mobilities of cultured flight sample cells

The observed electrophoretic mobilities in A-1 buffer of the cultured cells from sample slices 14, 17 and 19 agree well with the values predicted by equation [21] for cells at their final locations in the flight column:

<u>Slice No.</u>	<u>U_e²⁵ in A-1 buffer</u>	
	<u>Observed*</u>	<u>Predicted†</u>
14	1.44 ± 0.02	1.30-1.36
17	1.56 ± 0.03	1.50-1.61
19	1.58 ± 0.02	1.68-1.76

* Mean ± standard error

† Range

While the agreement indicates that the EU performed as expected for this column, the evidence is not direct since the reclaimed cells were passed through two culture steps prior to measurement of their mobilities. Thus it is possible that the characteristics of the mobility distributions were altered, which may account in part for the dispersity of the mobilities observed for each of these samples (Fig. 3).

2.6.3 Examination of bases for disagreement between predicted and observed migration behavior

The major discrepancy between the predicted and observed migration behavior for kidney cells in the flight experiment is the large cell recovery in the vicinity of the sample slide, i.e., about 30% of the cell load was recovered from regions of the column where none were expected. All of these cells should theoretically have been recovered at larger migration distances corresponding to sample slice numbers 13 through 24 (see Table 1). At present it is not possible to identify with certainty the source of this discrepancy. However, a number of factors were considered and many have been judged to be implausible explanations.

The first question posed was whether the analytical mobility data base for the predictions (Figure 1) was in error. This data had been obtained for cells from a different primary cell line from that used for the flight experiment. In addition, the harvest procedure did not involve trypsinization of the cell

monolayers as did the preparation of the flight samples. Instead the harvest had involved mechanical removal of the cell monolayers from the culture flasks and disruption of the monolayers to single cells by repeated aspiration of the cell suspension through a narrow channel (Pasteur pipet tip). The suspensions prepared in this fashion contained significant quantities of fibrous debris and a number of rapidly sedimenting cell aggregates. Thus it was possible that very low mobility cells may not have been detected if they had sedimented rapidly as individual cells or in cell aggregates. These points were checked during postflight analyses (Table 2).

Mobility distributions for whole populations of kidney cells differed little for cells from different primary cell lines or as a consequence of trypsinization (Table 2, Exp. nos. 1-4). In experiment 5 (Table 2) cell monolayers were scraped from the surface of the culture flasks following removal of the flask sides with a hot knife. The monolayer pool was split and one half was trypsinized while the second was repeatedly aspirated in order to disperse the cells. Cell viability (Trypan Blue dye exclusion) was virtually lost in the sample dispersed by mechanical means, while one third of the trypsinized cells were still viable. It was suspected that the low viabilities shared by both samples were associated with exposure of the cells to noxious fumes arising from the removal of the flask sides. The retention of viability in the trypsinized sample and lack of mobility differences following trypsinization indicated this to be the preferred harvest procedure. Associated with the low viability was a several per cent decrease in the mean mobility of the whole populations with indications of a general broadening of the distribution. No extremely slow cells or aggregates were observed.

In experiment 6, trypsinized cells from experiment 4 were rate frozen in A-1 buffer and rapidly thawed. Without any washing of the thawed cells, their mean mobility was about 14% less than that of the control cells, but following one wash with A-1 buffer their behavior returned to about control values.¹ Slow freezing of the cells to -20°C and slow thawing, conditions which lead to substantial cell damage and loss of viability, markedly increased the tendency of the cells to aggregate. Only a limited number of mobility measurements were obtained which indicated a small decrease in mean mobility, but neither individual cells nor aggregates were observed with mobilities approaching zero.

The general conclusions of these studies of various treatments on the mobilities of kidney cells in A-1 buffer were:

- a. There was no evidence that errors in the original mobility data were responsible for the discrepancies between the predicted and observed behaviors in the flight experiment. Different harvest procedures and primary donor sources had little influence on the electrokinetic characteristics of the whole kidney cell populations and there were no indications that very slow subpopulations had been overlooked due to sedimentation problems.
- b. No evidence could be obtained which supported the source of the discrepancy as a substantial reduction of the mobilities of kidney cells due to cell damage. Loss of viability resulting from cell

¹ Washes conducted at room temperature, ~ 22°C.

TABLE 2. MOBILITY DATA FOR WHOLE POPULATIONS OF KIDNEY CELLS IN A-1 BUFFER FOLLOWING VARIOUS TREATMENTS

Cell Lot	Exp. No.	Treatment	% Viab.	Mobility, U_e^{25}	
				Mean $\pm$ SD (n)	Range
995-1	1	Harvest without Trypsin.	ND	1.66 $\pm$ 0.16 (200)	1.32-2.12
995-2	2	Harvest without Trypsin.	ND	1.67 $\pm$ 0.19 (95)	1.34-2.12
915-2	3	Harvest with Trypsin	80-90	1.60 $\pm$ 0.19 (73)	1.11-2.34
1201-2	4	Harvest with Trypsin	95	1.61 $\pm$ 0.16 (100)	1.28-2.24
915-2	5	Harvest without Trypsin	~0	1.48 $\pm$ 0.32 (50)	0.92-2.53
915-2	5	Harvest with Trypsin	33	1.48 $\pm$ 0.22 (50)	1.13-2.29
1201-2	6	Harvest with Trypsin. Rate frozen in A-1.	58	1.38 $\pm$ 0.11 (38)	1.16-1.52
1201-2	6	Harvest with Trypsin. Rate frozen in A-1, rewashed.	58	1.58 $\pm$ 0.11 (30)	1.34-1.80
1201-2	6	Harvest with Trypsin. Slow freeze-thaw.	0	1.52 $\pm$ 0.20 (15)	1.27-1.85

damage during freezing or adverse harvest conditions failed to produce large decreases in electrophoretic mobility. Associated with viability loss were: small decreases in the mean mobility; the appearance of debris in the sample suspensions which included 5-10 μm vesicles with mobilities greater than those of the parent kidney cells; and a markedly increased tendency for the cells to adhere to one another as well as to surfaces. The only treatment found which produced extremely slow cells was low suspending medium pH ($\sim$ pH 4) as described in section 3.3. However, there was no evidence for the occurrence of such low pH's in flight column 3.

Another major potential source of the discrepancy was malfunctioning of one or another of the components of the electrophoresis unit. Malfunctions were responsible for aberrant cell migrations in columns 2 (lymphocytes), 5 (red cells), 6 (lymphocytes) and 7 (kidney cells) (1,12). In column 2 a fluid line blockage resulted in bubble formation in the electrode chamber which blocked the current path and subsequently prevented any cell migration. In columns 5 and 6 restriction of the buffer purge of the column electrode housings led to accumulation of electrolysis products in the electrophoresis columns which significantly altered the pH of the electrophoresis buffer. These pH alterations were demonstrable in the sample slices during post flight examination (1,2,12). As noted earlier, the operation of column 7 was aborted due to a fluid line leak.

There were no indications in the flight log, in comments by the operators of the flight experiment, or in the post flight examinations of the equipment that malfunctions occurred in the column 3 experiment. The pH's of the sample slices ranged from 7.0-7.9 (Table 1). Column voltages were only recorded for 15' due to problems with film exposure but during that time the column voltage remained in a reasonable range (204-218 volts) and the migration behavior of the bulk of the kidney cell population (Figure 4) suggested that this condition persisted through the entire 75 minute run.

It was also possible that electroosmosis had influenced the migration behavior of the kidney cells. If the electrostatic charge on the column wall were negative due to for example incomplete coating of the column with methyl-cellulose or adsorption of negatively charged materials from solution, then the resulting electroosmotic fluid flow would be toward the sample slide for the buffer near the column wall. Return flow in the direction of kidney cell migration would occur in the center of the column in the absence of fluid flow through the agar impregnated sintered glass discs which separated the column buffer compartment from the electrode compartments. The expected results of such a closed system flow would be a pronounced broadening of the expected distribution profile shown for no electroosmosis in Figure 4A. Also, the central region of the distribution would be shifted to larger migration distances if the sample cells remained most concentrated near the radial center of the column where they entered the column from the sample slide.

It is clear from the relationship between cell migration and electroosmosis (equation 21) that the fine details of the final cell distribution profile in the presence of electroosmosis depend largely on the radial location of the cells in the column. There is no available information on

the radial migration behavior of the cell population once the cells entered the column.

In general, there were no clear indications of problems with electro-osmosis in column 3. The coating procedures were effective for all of the columns employed in the flight experiment as evidenced by direct examination of each prior to loading for the flight experiment (11). The final cell distribution in the column (Figure 4B) could be interpreted as broadened, but the preponderance of cells at very small migration distances is not balanced by significant numbers of cells at very large migration distances as might be expected if the electroosmotic mobility in the column were comparable to the electrophoretic mobilities of the cells, i.e. the condition where approximately zero net migration would be observed for a cell located at the column wall.

During the sample slicing operation no problems were noted which could account for the observed distributions. It does not appear plausible that the large numbers of cells in question were displaced by cross contamination during the slicing operations.

Overall, factors have been identified which can explain small discrepancies between the predicted and observed behaviors. However, none have been found which can be linked with any certainty to the larger discrepancy where very little migration was observed for a significant portion of the cell population. The high correlation between growth response of the recovered cells (Figure 4B) and their predicted location in the column suggested that loss of cell viability was involved. As noted earlier no evidence could be obtained for a large reduction in electrophoretic mobility as a consequence of a loss of cell viability. However, it was observed that increases in cell adhesiveness were associated with cell damage so that it is possible that this characteristic of the cells was involved in hindering their migration in the column. Since there was no evidence in any of our post flight studies for cell aggregates of nearly zero mobility, hindrance of cell migration resulting from increased cell adhesiveness would have to involve contact with the surfaces of the sample slide and/or the column. Small misalignments of the sample slide in the column or complex fluid circulations in the sample slide could plausibly provide the conditions for such contacts. It is difficult to assess the plausibility of this hypothesis since too many factors are involved. For example, cell leakage products resulting from cell damage could coat the sample slide surface providing an electroosmotic flow in the sample slide which would interact in some way with the adjacent column fluid compartment. Also, the smaller diameter of the sample slide constricted the electrical field and provided a region where Joule heating was greater than in the column and heat sinking was probably less efficient due to the geometry about the slide. However, the bulk of the evidence examined pointed to such a hindrance of cell migration as opposed to modification of the mobilities of the cells or unexpected fluid flows in the electrophoresis column.

3. KIDNEY CELL EXPERIMENTS PERTAINING TO FUTURE ELECTROPHORETIC EXPERIMENTS

3.1 Background

During the planning stages for the ASTP MA-011 experiment a single electrophoresis buffer was to be identified for use in the electrophoresis column and buffer reservoirs for all samples in the zone electrophoresis experiment. The A-1 buffer formulation was adopted since it was compatible with the flight hardware as well as with the candidate cell samples for the experiment. The most sensitive cell samples were the lymphocytes whose viability was more sensitive to environmental factors than the kidney cells.

In this chapter, an alternate buffer for any future electrophoresis experiments involving HK cells is examined and compared with A-1 buffer. The buffer formulation was suggested by Abbott Laboratories as a more suitable buffer for electrophoresis of kidney cells based on pilot electrophoretic studies which showed that kidney cells had substantial mobilities in the buffer and could be recovered with minimal decreases in viability following electrophoresis. No data, however, were collected to establish whether cell viability was significantly different for cells frozen in this buffer with added cryoprotectant (glycerol) when compared with A-1 buffer. In addition data on cell sizes, and the dependence of mobility on suspending medium pH were collected.

3.2 Alternate Electrophoresis Media

3.2.1 Formulation and properties

The following formulation excluding glycerol was proposed by Abbott Laboratories as an alternate electrophoresis medium. For experiments involving freezing of cells in the buffer, glycerol was added as a cryoprotectant at the same concentration as employed in A-1 buffer. The buffer in the absence of glycerol will subsequently be designated as buffer A and that containing glycerol, buffer AG:

<u>Substance</u>	<u>M. Wt.</u>	<u>g/liter</u>	<u>Millimolarity</u>
Glucose	180.16	43.38	240.8
Na ₂ HPO ₄ ·7H ₂ O	268.08	3.433	12.80
KH ₂ PO ₄	136.09	0.2006	1.474
Glycerol	92.11	47.3	514.

Both buffers were characterized by the same methods used in the description of A-1 buffer (2). The previously reported properties of A-1 buffer are included for comparison.

	Buffer		
	<u>A</u>	<u>AG</u>	<u>A-1</u>
pH	7.76	7.80	7.28
Osmolality (mOsm/Kg)	283	877	847
Density (g/cc @ 25°C)	1.0145	1.0255	1.0223
Viscos., η (cPoise @ 25°C)	1.02	1.13	1.11
Conductivity, κ (mmho/cm @ 25°C)	2.09	1.85	0.95

The most significant differences are in the pH and conductivity of the proposed buffers, both being higher than the values for A-1.

3.2.2 Behavior of kidney cells in the alternate media

The electrophoretic mobilities for various unfractionated kidney cell populations in A-1, A and AG buffers are summarized in Table 3. All of the cell populations were harvested from confluent cultures.

In the first experiment the cultures had been maintained for ~ 5 days at 37°C in maintenance medium. Following harvesting with trypsin cell viability was $\sim 90\%$. Cell samples in each of these media as well as the phosphate buffer wash medium were examined in an Electrozone/Celloscope electronic particle sizing apparatus in order to provide characteristic population cell size values. The modal volumes of the cell samples in all of these buffers were ~ 1700 to $2000 \mu\text{m}^3$ (range 1100 to $3100 \mu\text{m}^3$) which corresponds to a spherical diameter of $\sim 15 \mu\text{m}$. Differences were noted between the values for the populations in buffers containing glycerol (A-1 and AG) as opposed to those containing no glycerol (A and buffered phosphate wash medium). In the former, the modal cell volumes were approximately 10% lower. Also, the upper end of the cell number-cell volume distribution curve occurred at approximately a 10% lower value than those for the cells in buffers not containing glycerol while the lower ends of the curves showed little displacement.

In experiments 2 and 3 control mobility values were obtained for cells at confluency in growth medium and harvested with trypsin for freeze-thaw experiments (exp. 3b and 3c). Viability was 95% prior to rate freezing and rapid thawing. The cells ($5 \times 10^6/\text{cc}$) were maintained for 30 minutes at the temperature of liquid N_2 .

Following rapid thawing the viabilities were 58% for cells in A-1 buffer and 53% for those in AG buffer. The mean mobilities of the cells in each thawed suspension were about 10-15% lower than the control values but returned to essentially control values following one wash in the given electrophoresis medium.

In experiment 4 it was found that trypsinization of EDTA harvested cells had little effect on the mobilities of the cell populations. Cells harvested with EDTA were much more adhesive and cell aggregation was pronounced in comparison with trypsinized cells.

Experiment 5 was an examination of the mobilities of cells which had been cultured in urokinase production medium for different lengths of time. The

TABLE 3. COMPARISON OF MOBILITIES OF WHOLE KIDNEY CELL POPULATIONS IN VARIOUS BUFFERS AT 25°C

Exp. No.	Cell Lot	Treatment	Mean Mobility $\pm$ SD (no. measurements)		
			Buffer A	Buffer AG	A-1 Buffer
1	915-2	Maint. Med. - Trypsin	1.50 $\pm$ 0.19 (75)	1.25 $\pm$ 0.11 (75)	1.60 $\pm$ 0.19 (73)
2	1201-2	Growth Med. - Trypsin		1.32 $\pm$ 0.14 (70)	
3a	1201-2	Growth Med. - Trypsin		1.21 $\pm$ 0.11 (100)	1.61 $\pm$ 0.16 (100)
3b	1201-2	Freeze-thaw - No wash		1.14 $\pm$ 0.11 (30)	1.38 $\pm$ 0.11 (38)
3c	1201-2	Freeze-thaw - 1X wash		1.25 $\pm$ 0.14 (30)	1.58 $\pm$ 0.11 (30)
4a	1201-2	Growth Med. - EDTA	1.49 $\pm$ 0.16 (40)		
4b	1201-2	Growth Med. - Trypsin	1.43 $\pm$ 0.12 (40)		
5a	915-3	31 day Prod. Med. - Trypsin	1.41 $\pm$ 0.13 (100)		
5b	915-4	17 day Prod. Med. - Trypsin	1.44 $\pm$ 0.10 (50)		

intent was to establish whether any substantial shifts in the mobility distribution resulted from this change in the culture conditions. The "control" cells were those in experiment 1 which were transplanted to culture in production medium at the third cell passage for the 31 day culture, while part of the lot was recultured in growth medium and then transferred to production medium at the fourth passage. There was a trend toward a lower average population mobility with increasing time in production medium. Associated with this trend was a narrowing of the mobility distribution. For lot 915-2 the range was 1.06-2.11 mobility units while those for 17 and 31 day cultures in production medium were 1.29-1.79 and 1.16-1.69, respectively. Additional studies would be required to establish whether this small mobility shift is the result of transfer to production medium or might be explained simply by progressive changes in the cells during prolonged periods of tissue culture which would occur even in growth medium.

3.3 Dependence of Kidney Cell Mobility on Suspending Medium pH

Previously no data had been collected on the pH dependence of kidney cell mobility. Thus it was not possible to predict the extent to which pH drifts in the electrophoresis medium would alter the mobility of kidney cells. Mobility data were collected (Figure 5) for kidney cells from two of the cultures employed in the collection of the data in Table 3. The trypsinized cells were suspended in a simple ionic medium, 0.03 M NaCl, to which sorbitol was added (4% w/v) in order to maintain proper medium tonicity. Exposure of the cells to pH's from 5-10 for ~ 30 minutes at room temperature in this medium produced no irreversible changes in electrophoretic mobility, i.e., readjustment of the pH to 7.2 ± 0.2 gave control mobility values. Following exposure to pH's less than 5, the mobilities of the cells upon readjustment to ~ pH 7 were ~ 10% higher than the control values and the suspension contained debris indicative of cell fragmentation. As can be seen from Figure 5, the mobility of the kidney cells increases continuously through the pH region from 6 to 8 but the increase is small. Substantial changes in mobility occur at pH's greater than 9 and less than 6. This behavior is consistent with the anticipated pK values for the ionizations of amino and carboxyl groups, respectively (8).

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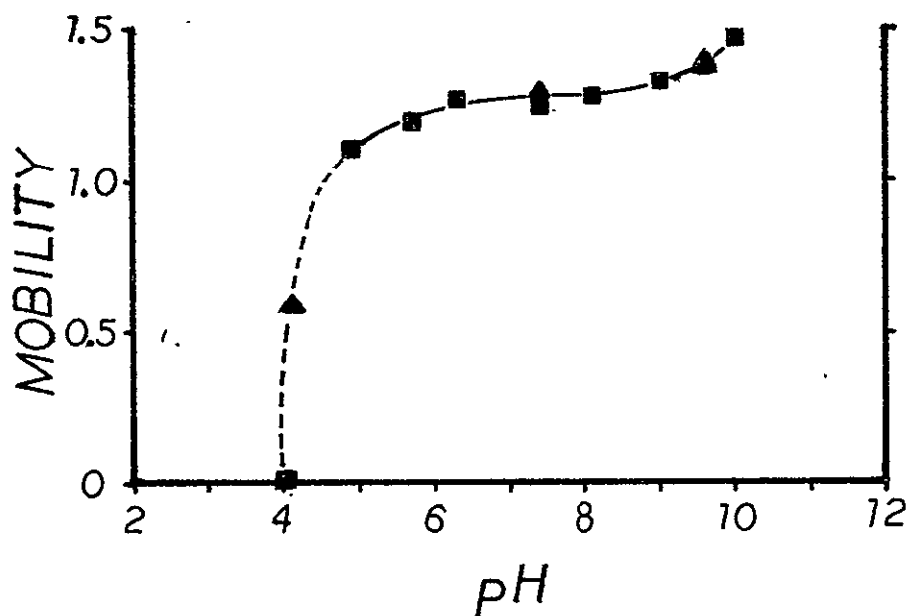


FIGURE 5. Dependence of Kidney Cell Electrophoretic Mobility on Suspending Medium pH. Mean electrophoretic mobilities ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) at 25°C are plotted for whole populations of kidney cells suspended in 0.030 M NaCl-4.0% w/v sorbitol: $\square$ cells from lot 915-3 cultured 31 days in production medium; Δ cells from lot 915-4 cultured for 17 days in production medium. Each point represents the mean of ~ 10 measurements. All cells were harvested with trypsin and washed in phosphate buffer prior to transfer to the low ionic strength medium. Media pH's were adjusted to approximately the desired values with 0.03 M HCl or 0.03 M NaOH prior to addition of kidney cells and the final values were measured 2-5 minutes after the addition of the cells. The dashed portion of the curve represents the pH region where the mobilities of the cells were not reversible upon readjustment of the pH to 7.2 ± 0.2 .

4. STANDARD PARTICLES FOR ELECTROPHORESIS

4.1 Background

Electrophoresis of small particles (1-30 μm) is currently employed primarily as an analytical tool in a variety of contexts where a description or determination of particle surface charge density is of interest (8,13). At present the application of the method to problems of physical separation of discrete electrophoretic populations of particles has been limited primarily due to technical problems in the development of suitable equipment. However, recent interest in the application of electrophoretic techniques to the study of small particles has provided the impetus for developing more suitable instrumentation for both analytical and preparatory applications. One problem area which has been emphasized by these efforts is the lack of suitable electrophoretic standard particles which may be used in assessing instrument performance.

One of the most commonly used "standard" particles in analytical electrophoretic applications is the red blood cell (8). A substantial body of data has been accumulated in different laboratories which provide certain values for the mobility of e.g. human red blood cells under specified conditions (8). This information also indicates that nature exercises exquisite control over the dispersity of the mobility distribution for human red cells from healthy donors so that the particle mobility distribution is monodisperse within the experimental limits of the measurement systems used to date. Moreover, different species provide red cells of differing size, shape, and electrophoretic mobility.

The major shortcoming of fresh red blood cells as standard particles is their fragility, i.e. the range of conditions under which they are stable and the limited duration of their stability. While they can be viewed as a readily available source material for "primary" standardization of analytical electrophoresis equipment, their use is not practical for many workers who do not have the necessary collection and processing equipment in their laboratories. However fresh red cells do offer considerable promise as the raw material for chemical modifications which are designed to render them stable as well as those aimed at alteration of their mobilities to desired values.

The objectives for this second phase of the contract period involved utilizing available knowledge in the research literature in the production of particle standards for electrophoresis from red blood cells. The specific project aims were as follows:

- Screen the most potentially useful chemical modification techniques for stabilizing and selectively modifying the mobilities of candidate red cells.
- Test chemical modification techniques and establish whether these can be used reliably to produce acceptable particle populations whose electrophoretic properties are stable for acceptable time periods under conditions which will be defined.

- Provide a standard protocol for testing and acceptance of standard particle preparations.
- Provide representative mobility data for standard particle preparations in simple ionic media at different pH's and ionic strengths.

4.1.1 Particle characteristics of interest

A review of the general needs for the NASA electrophoresis program and or users involved in studies of biological particles indicated that the following characteristics should be considered for candidate standard particle preparations:

Electrophoretic properties:

- Essentially monodisperse electrophoretic mobility distribution.
- Different candidates should span the mobility range commonly encountered for biological cell populations, i.e. ~ 0.5 to 4×10^{-4} $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ at ionic strengths of ~ 0.01 M.
- Stability: the mean mobility and dispersity should be stable for the particle preparation for a definable time period under specifiable storage conditions. Preferably the period of stability would be greater than about 3 to 4 months.

Physical properties:

- Size: ~ 1 - 20 μm for major dimension.
- Density: preferably not more than 10% greater than that of H_2O so that the particles will not sediment at excessive rates.
- Refractive index: ~ 1.40 or greater if particles are transparent and colorless so that they are observable with standard bright field microscope optics.
- Shape: no particular shape preferred although distinctly different shapes for different candidates could be used for their differentiation in mixtures.
- Color: same consideration as for shape with regard to differentiation of particles in studies of resolution.

Chemical properties:

- Hydrophilic for use in aqueous media so that adsorption of potential surfactants will be minimized.
- Chemically stable during storage life under specified conditions.

- Functional groups: modifiable functional groups make it possible to tailor the electrophoretic properties to the needs of the user.

Availability:

- Particles should be available in reasonable quantity for any required modification steps and should require minimal chemical manipulations.
- Modification agents should be relatively common chemicals which are readily obtainable at satisfactory levels of purity.

In addition to these features it would be useful to have standard particles which could be stored in dry form thereby increasing storage life and minimizing the need for special washing procedures prior to their use.

4.1.2 Objectives and general experimental approach

Since the contract period for these studies was relatively short emphasis was placed on testing of those procedures which appeared most promising in solving certain aspects of the particle standard problem. These aspects included: the stabilization of the cells, controlled modification of cell size or density, treatments of cells to eliminate cell aggregation upon drying and modifications which alter the electrophoretic mobilities of the cells. All of the initial studies were conducted with freshly drawn human red blood cells since they are readily available and an extensive literature is available on their properties (8). Large quantities of outdated human red cells are available from the American Red Cross Blood Banking program so that this was a potential source for cells if these cells were found to have acceptable properties. Thus emphasis was placed on exploring the limits of the use of human red cells as a source material and developing the basic methodologies with human RBC prior to working with cells from other species.

4.1.3 Selection of candidate cell modification procedures

Many modification procedures are available for cells which provide alterations in one or more of their properties. Consequently, the selection of a specific set of procedures depends on the requirements of the user. The literature on red cell modifications is vast. However, a number of reviews deal with various general aspects of cell modifications which have been employed in studies of red cells. Cell fixation procedures have played a vital role in the field of histochemistry and recent reviews are available (14,15). Specific enzymatic alterations of the surfaces of cells have been employed as probes of surface structure (8,13,16) and certain enzymes such as neuraminidase and proteolytic enzymes such as trypsin or pronase may be used to modify the mobilities of native red cells (8,16,17). Specific chemical modifications have been employed which alter the mobilities of red cells but a major problem is that the required conditions necessitate prior stabilization or fixation of the cells (8). There is, however, a wide variety of potentially useful chemical modifications largely derived from studies of proteins and these have recently been reviewed (18,19).

The cell treatments examined during the course of this contract were dictated in part by basic considerations of the features deemed desirable for single standard particle preparations for electrophoresis and in part by additional properties which were to be modified for the formulation of test mixtures of particles. In the latter case particle mixtures were required for testing resolution in electrophoretic separation devices such as the continuous particle electrophoresis apparatus or the electrophoretic separator in the SPAR program (20). These particle mixtures were to be composed of two to three particle subpopulations which differed in their electrophoretic mobilities as well as in some other property such as size or volume which could be employed in nonelectrophoretic tests of the resolution of the subpopulations by the device in question.

The cell modification objectives considered during this work and corresponding candidate treatments are summarized in Table 4. Of the listed objectives, stabilization of the cells is the most fundamental to all uses of red cells as standard particles. Native red cells lyse during storage or under conditions of low pH or ionic strength (21). Since both storage and media of low ionic strength are often encountered in flight and ground-based experiments (20), stabilization procedures are required in order to remedy this susceptibility so that the electrokinetic properties of the cells will be stable for the duration of experiments which may last for up to one or two weeks.

A screening of the literature and our previous experience indicated that aldehyde treatment was the stabilization method of choice. Although tannic acid, osmium tetroxide and potassium permanganate have been used as red cell fixatives, these methods suffer deficiencies. All three of these agents do not stabilize the red cell to osmotic and chemical shocks to the extent that aldehydes do (25-27) which is attributable primarily to the ability of aldehydes to crosslink hemoglobin in the cell to form a gel network. There is little information available on the electrokinetic properties of cells fixed with these agents. In the case of tannic acid, variations in other surface properties of the fixed red cells have been attributed to variable tannic acid composition (33). Thus, since tannic acid represents a family of related compounds, spurious electrokinetic results are a more probable consequence. Osmium tetroxide was not adopted since in addition to the problem with cell stability, the reagent is very expensive and extremely toxic. While expense and toxicity are not problems with potassium permanganate, poor reproducibility in electrokinetic properties for different cell preparations has been observed (32). It is also probable that long term stability of permanganate treated cells would require additional treatments - as for example aldehyde fixation.

An interesting feature of permanganate and osmium tetroxide fixed red cells is the independence of their mobilities on ionic strength at less than ~ 0.1 M NaCl in contrast to the behavior of normal cells (26,27). This behavior has been attributed to an increased membrane conductivity due to the fixative (27). Consequently, where media of ionic strengths less than ~ 0.1 are to be used, the mobility decrement resulting from osmium tetroxide or potassium permanganate fixation could be useful.

TABLE 4. CANDIDATE MODIFICATION METHODS FOR ALTERING
VARIOUS PROPERTIES OF RED BLOOD CELLS

Objective	Treatment	Reference
Stabilization of lysis	Fixation in aldehydes	22-24
	Fixation in tannic acid	25
	Fixation in osmium tetroxide	26,27
	Fixation in potassium permanganate	27
Decrease of mobility	Neuraminidase	16,17,28
	Methanolic HCl	24
	Diazomethane	24
	Proteolytic enzymes ^a	16,17
Volume alteration	Aldehyde fixation in hypo- or hypertonic media	23
Decrease of density	Cell lysis	29-31
Dry fixed cells	Chloroform methanol extraction	

^a Proteolytic enzymes do not consistently give decreased mobilities for red cells from different animals and have produced increases for cat red cells (17).

Formaldehyde was selected for cell stabilization in the studies reported here since it is available in a highly purified nonvolatile form as para-formaldehyde which has previously been used in the fixation of red cells (22,23). The properties and chemistry of formaldehyde has been extensively reviewed (34). Acetaldehyde and glutaraldehyde were not employed since redistillation is often required in order to obtain preparations of adequate purity, especially for glutaraldehyde which polymerizes easily (15). Also, glutaraldehyde fixation introduces an anomalous "hump" in the pH mobility profile for human red cells (23) for reasons which remain unexplained.

Alterations of red cell mobility by chemical or enzymatic treatments provide a potential means of spanning a broad mobility range and greater flexibility in the preparation of standard particle mixtures. Red cells from different species span a large range of size and volume (35) as well as different morphologies. Consequently, the combination of inherent differences in electrophoretic and nonelectrophoretic properties with the ability to further manipulate the electrokinetic properties of the cells ultimately provides a large set of combinations of particles which could be used as test mixtures for electrophoresis. The enzyme neuraminidase cleaves sialic acid from the surface of erythrocytes thereby reducing the negative surface charge of the cells since the carboxyl group of sialic acid is a major contributor to the surface charge of red cells from many species (8,16,17,28). The degree of mobility reduction is variable for red cells from different species with maximal decreases for human red cells (16,17,28). However, although human cells of nearly zero mobility have been produced, subsequent formaldehyde fixation increases their mobilities to about 40% of control native cell values (36). Human red cells with zero mobilities have been prepared through esterification of cell surface carboxyl groups with methanolic-HCl or diazomethane (24) but these procedures require prior aldehyde fixation of the cells to prevent hemolysis. Proteolytic enzymes generally produce variable but smaller decreases than neuraminidase in the mobility of native red cells by cleaving cell surface peptides which bear anionogenic groups. However, in the case of cat red cells, protease digestion increases their mobilities by up to 35% (17).

Modifications of cell volume and density with accompanying electrophoretic studies are not available in the literature. The ability to control the volume and density of fixed cells was considered useful in manipulating nonelectrophoretic properties of cells for the production of particle mixtures. Also, reductions of cell density and cell size were anticipated to reduce the sedimentation rate of fixed cells thereby prolonging the time for electrophoretic examinations. Native human red cell volumes may be manipulated over a finite range by changing the tonicity of the suspending medium (37-39) and volume differences are maintained following aldehyde fixation (23).

A major determinant of red cell density is the cell hemoglobin concentration, which generally is ~ 30 g/100 cc for mammalian red cells and accounts for the cell having a density of ~ 1.1 g/cc which is about 10% greater than that of physiological saline (0.9% w/v NaCl). Hemoglobin is also responsible for the color which makes red cells visible by standard bright field microscopy. It was anticipated that by lysing the cells under hypotonic conditions it was possible to reduce the hemoglobin concentration

so that the cell density would approximate to a density of ~ 1.0 at 25°C and yet retain enough color to be visible by bright field microscopy

The last objective - a dry fixed cell preparation afforded the potential advantage of a prolonged shelf life. In media with ionic strengths greater than ~ 0.03 , formaldehyde fixed red cells aggregate weakly (40), and this has been attributed to van der Waals forces. Pilot experiments conducted in this laboratory indicated that drying of fixed cells suspended in dilute salt media produced a dry cake which could not be redispersed. Lipid extraction afforded the possibility of decreasing long range van der Waals forces thereby decreasing the strength of aggregation of the cells upon drying.

4.1.4 Selection of red cells

Red blood cells from different species differ in their electrokinetic behavior (8), size and volume (35), susceptibility to osmotic lysis (41) and many other properties. However, the bulk of the literature on red cell properties and behavior deals with human red cells. For this reason, human red cells were employed for most of the studies during this contract since the extensive literature provided useful information for interpreting their behavior. Also, these cells are the most readily available in sufficient quantity for generating particle preparations.

Cat and dog red cells were viewed as candidates for preparing particles with large mobilities since their mobilities are larger than those for other species of red cell under standard test conditions (8,16). In addition, proteolytic treatment of cat cells increases their mobilities (17). However, cat cells are not generally available in adequate quantities and the extent of intraspecies variation in their electrokinetic and other properties is not established. Dog cells were not employed since these are much more prone to hemolyze during cell modification procedures (32) such that fixed cell preparations displaying a wide range of electrophoretic mobilities have been obtained (2).

Rabbit and goat red cells were selected for pilot studies since they afforded potential advantages as standard particles for the production of test mixtures. These cells have much smaller volumes than human cells: $\sim 55 \mu\text{m}^3$ for rabbit, and $\sim 15 \mu\text{m}^3$ for goat in contrast to $90 \mu\text{m}^3$ for human. Also, rabbit red cells have the lowest mobility of any mammalian red cell studied to date (8). However, there was no information available on goat cells.

4.2 Experimental Methods

4.2.1 Analytical particle electrophoresis

Analytical particle electrophoresis was conducted as described in 2.4.1. The electrophoresis chamber was calibrated with fresh human red cells and its operation considered satisfactory when the mean mobility of ten or more

measurements was 1.08 ± 0.03 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ for cells in 0.15 M NaCl or 2.40 ± 0.06 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ for cells in 0.015 M NaCl-4.7% w/v sorbitol (both media adjusted to pH 7.2 ± 0.3 with NaHCO_3). Coefficients of variation larger than about 8% for fresh red cells were considered spurious since their mobility distributions are narrow and appear to be limited by the accuracy of the measurement device (42,43). The in vivo age of human red cells does not broaden their mobility distribution (42,43). Larger coefficients of variation are anticipated for lower mobility cells where measurement errors due to electroosmotic fluid flow and proper focus of cells become more significant (44).

4.2.2 Collection and washing of red cells

Blood was obtained by venipuncture from healthy adult humans, American-Dutch rabbits, and two female Pigmy goats. The bloods were immediately mixed with 1.5-2.0 mg disodium (ethylene dinitrilo) tetraacetate dihydrate ($\text{Na}_2\text{EDTA} \cdot 2 \text{H}_2\text{O}$) per ml of whole blood in order to prevent coagulation (45). Units of human whole blood with citrate phosphate dextrose as anticoagulant (46) were obtained from the Northwest Regional Center of the American Red Cross. Fresh units were obtained as "possible air contamination" and outdated units had been stored at 4°C for twenty-one days or longer.

Fresh human blood samples were washed and fixed in aldehyde (see below) on the day of collection. Rabbit and goat blood and human blood obtained from the Red Cross were stored at 4°C for up to five days prior to use.

A standard wash procedure was used to prepare red cells for electrophoresis, or any modifications performed on fresh cells. This consisted of centrifuging the whole blood sample at 1500-2000 $\times g$ for 20 min at room temperature. The supernatant plasma and the buffy coat (thin yellowish-white layer of white cells and platelets) were aspirated. The red cell pack was thoroughly suspended in standard saline (0.15 M NaCl adjusted to pH 7.2 ± 0.2 with 0.15 M NaHCO_3) using ~ 10 or more volumes of saline per volume of cell pack and the suspension was centrifuged as above. The supernatant saline was aspirated and discarded. This wash procedure was conducted a total of three times when small volumes of cells were being processed and the volume ratio of saline to packed cells exceeded ~ 30 . Where the ratio was only about 10 a fourth wash was performed in order to assure removal of plasma constituents. Red cells carried through this wash regimen will subsequently be referred to as washed red cells.

At each washing care was taken to remove residual buffy coat so that the final preparation would contain negligible numbers of platelets or leukocytes. Residual aggregated material at the bottom of the centrifuge tube was also separated from the suspended red cells by decantation of the latter.

During these wash procedures the supernatant saline should have very little red color which indicates hemolysis of the cells. Hemolysis can arise from the use of very old cells, a spuriously low saline concentration, simple mechanical trauma during resuspension procedures, or thermal shocks where the cells at one temperature are suspended abruptly in a wash medium which differs in temperature by $\sim 10^\circ\text{C}$ or more. For outdated Red Cross blood, some hemolysis

is seen during the first washes but progressively decreases.

A higher concentration of NaCl (0.207 M) is required for washing goat red cells since goat plasma has a much higher solute concentration (381 mosmoles/Kg by freezing point depression) than human or rabbit plasma. This will be discussed in section 4.3.

4.2.3 Preparation of formaldehyde fixative

The formaldehyde fixative was routinely prepared from powdered paraformaldehyde (Matheson Coleman and Bell, MCB) (23) and contained a calculated 1.5% w/v formaldehyde assuming that paraformaldehyde was composed only of polymerized formaldehyde (HCHO). This calculated value is several percent higher than the true value which is closer to 1.4% since the commercially available paraformaldehyde contains several percent water (34). The aldehyde was made up in a phosphate buffered saline (PBS) which is isotonic for human red cells (23).

For the preparation of the fixative and red cell wash media a 2x concentrated PBS stock is prepared as indicated below. Isotonic PBS is prepared by diluting 1 volume of 2x PBS with 1 volume of distilled H₂O.

<u>Substance</u>	<u>F.Wt.</u>	<u>2x prep.</u>		<u>1x final</u>
		<u>mMolarity</u>	<u>g/liter</u>	<u>mMolarity</u>
Na ₂ HPO ₄ ·7 H ₂ O	268.08	50.8	13.610	25.4
NaH ₂ PO ₄ ·H ₂ O	137.99	9.23	1.274	4.62
NaCl	58.44	240	14.026	120

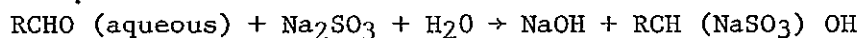
The pH of the 2x preparation is 7.4 while that of the 1x PBS is 7.5. The osmolality of the 1x PBS is 287 mosmoles/Kg as measured by freezing point depression.

For one liter final volume of 1.5% formaldehyde, suspend 15.0 grams paraformaldehyde in 500 ml of 2x PBS in a 750 ml Erlenmeyer flask. Warm and stir the solution on a magnetic stirrer-hot plate in a fume hood until the temperature has reached ~ 60°C and then maintain at 60-70°C for one half hour during which time the suspension should clear and display only a slight turbidity. Cool the solution to room temperature (an ice bath is suitable) and filter through Whatman filter paper into a 1 liter volumetric flask. Following passage of the last aldehyde solution through the filter, rinse through last traces of PBS and aldehyde with a 50-100 cc aliquot of distilled H₂O. Bring the filtrate volume to 1 liter with distilled H₂O.

The pH of the solution should not be more than 0.1 to 0.2 pH units lower than that of the 1x PBS. Fixative solutions should be stored at room temperature in sealed glass containers.

The amount of free aldehyde present in the fixative solution may be estimated by the sodium sulfite method (34). This procedure may also be used to estimate the amount of aldehyde remaining in supernatant fluids from red

blood cell suspensions undergoing fixation for various periods of time. The assay is based on the reaction:



The NaOH liberated may be titrated with standard HCl using thymolphthalien as an indicator.

$$\text{Percentage yield} = \frac{\text{Acid titer} \times \text{HCl normality} \times \text{aldehyde M.W.}}{\text{Sample weight} \times 10}$$

The percentage yield obtained for fresh formaldehyde fixative solutions is normally in the range 90 to 95% (34).

4.2.4 Red cell fixation in formaldehyde

Prior to fixation the washed red cells, or cells carried through other treatments followed by washing, were washed once in PBS (4.2.3) as described for standard saline (4.2.2). The red cell pack is then completely resuspended in enough formaldehyde-PBS solution to give a final packed cell to fixative volume ratio of 1:50.

The containers are tightly capped and stored at room temperature for at least 48 hours at which time they will be ready for use. The cells should darken within approximately several hours and appear brown at the end of ~ 24 hours. Any visible leakage of colored material into the supernatant fluid during fixation should be noted. If significant coloration is present discard the sample. It is advisable also to measure the pH of the supernatant fixative solution after 24 hours and prior to use of the cells in an experiment. Usually the pH is ~ 7 but if it is lower than 5.5 the cells should be discarded.

4.2.5 Trypsin and neuraminidase treatments

For enzyme treatments of native¹ red cells with trypsin (Worthington lyophilized, 180 U/mg) or *Vibrio cholerae* neuraminidase (Behring Diagnostics, 500 U/ml) washed red cells were suspended in 0.15 M NaCl-0.005 M CaCl₂ to a volume concentration of 17-23%. Crystalline trypsin was added at 1 mg/ml RBC (16) and the suspensions were incubated for 90-120 min in a 37°C water bath. Neuraminidase was employed at a level of ~ 100 U/ml RBC. The appropriate volume of enzyme solution as supplied by the manufacturer (500 U/ml) was mixed with the red cell suspension and followed by incubation at 37°C for 90-120 min to insure complete enzyme action (36).

Neuraminidase, in contrast to trypsin, may be used to modify formaldehyde fixed red cells. The sites of action for neuraminidase are not blocked by aldehyde treatment as are those for trypsin. Details of the kinetics of neuraminidase action on human red cells and assay of sialic acid release are in the literature (36).

Formaldehyde fixed red cells were centrifuged (10 min, 2000 xg) from the fixative and were washed twice in ten volumes of standard saline and once in

¹ "Native" designates red cells which have not been modified with enzymes or chemical treatments.

0.15 M NaCl-0.005 M CaCl₂ followed by suspension in the latter to a volume concentration of 10-20%. It should be noted that fixed cells are rigid compared with fresh cells and following centrifugation at ~ 2000 xg about 40% of the pack of fixed cells is intercellular fluid in contrast to ~ 5% for fresh cells. Neuraminidase was added to the suspensions to give the same final concentration as used above and the samples were incubated at 37°C for two-four hours.

Following incubation at 37°C, native or fixed cells were recovered from the suspensions by centrifugation for 10 min at ~ 2000 xg at room temperature and were washed twice in > 5 volumes of 0.15 M NaCl or PBS in order to remove the enzyme. The cells were then carried through the fixation procedure (4.2.4).

4.2.6 Osmotic lysis

Osmotic lysis of red cells was used to generate red cell "ghosts" which had much lower densities than native cells. Native human red cells have a mean density of 1.09 g/cc in isotonic media (42) and the difference between the densities of the cells and isotonic NaCl at 1.004 g/cc (25°C) is attributable to hemoglobin. This can be demonstrated by simple computation. The cell density, ρ_c (g/cc), may be expressed as:

$$\rho_c = \frac{m_s + m_h}{V_c} \quad [22]$$

where m_s and m_h are the masses in grams of intracellular saline and hemoglobin, respectively, in a volume V_c (cc) of cells. The mass of saline, m_s , may be computed from the mass of hemoglobin and the cell volume, V_c :

$$m_s = \rho_s (V_c - \bar{v}_h m_h) \quad [23]$$

where ρ_s is the density of saline (1.004 g/cc at 25°C) and $\bar{v}_h$ is the partial specific volume of hemoglobin (0.72 cc/g).

Substitution from [23] into [22] gives:

$$\rho_c = \frac{\rho_s (V_c - \bar{v}_h m_h) + m_h}{V_c} \quad [24]$$

Computation of ρ_c for a single red cell with a volume of 90 fl or 9×10^{-11} cc and a hemoglobin content of 30 pg gives a value of 1.096 g/cc which is in excellent agreement with measured values (42).

Solving [24] for hemoglobin concentration, m_h/V_c gives:

$$\frac{m_h}{V_c} = \frac{(\rho_c - \rho_s)}{(1 - \rho_s \bar{v}_h)} \quad [25]$$

which may be used to compute the hemoglobin concentration corresponding to a given cell density. In principle these hemoglobin concentrations can be obtained by osmotic lysis of a defined volume of cells in a defined volume of lysis medium. If all cells lyse, the hemoglobin is distributed equally through the total fluid volume in the suspension. Upon lysis, the osmotic stress is relieved and the red cell membranes reseal (31) so that the external hemoglobin may be removed by standard wash procedures. Employing the hemoglobin concentrations computed for various final cell densities with equation [25] one can estimate the required volumes of cells, V_c , and lysis medium, V_l :

$$V_c C_h' = (V_c + V_l) C_h \quad [26]$$

where C_h' is the native red cell hemoglobin concentration in g/cc and C_h is the hemoglobin concentration following lysis.

Solving [26] for V_l gives:

$$V_l = V_c \left[\frac{C_h'}{C_h} - 1 \right] \quad [27]$$

The following table provides an idea of the relationships between cell density, hemoglobin concentration, and the volumes of lysis medium required to obtain different cell densities.

Cell Density ρ_c (g/cc)	Hemoglobin ^a Concentration (g/100 cc)	Lysis Medium ^b Volume (cc)
1.096	33.2	0
1.090	31.0	0.07
1.080	27.4	0.21
1.070	23.8	0.39
1.060	20.2	0.64
1.050	16.6	1.00
1.040	13.0	1.56
1.030	9.4	2.54
1.020	5.8	4.75
1.010	2.2	14.3

^a Calculated with equation [25] and $\bar{V}_h = 0.72$ cc/g, $\rho_s = 1.004$ g/cc.

^b Volume required for 1.0 cc of packed red cells.

These estimations are useful general guides but it should be remembered that the volume of red cell ghosts may change in response to medium solute concentrations and other conditions (30). The major problem with the osmotic lysis is technical in nature with regard to achieving the same final hemoglobin concentrations for all cells. No matter what medium is used, ideally

lysis must occur for all cells at the same time in order to obtain comparable equilibration for all the cells.

The two osmotic lysis procedures tested employed washed human red cells. In the first procedure, the hematocrit was measured for the red cell pack in order to determine how much residual standard saline was in the pack. An aliquot of cell pack was weighed into a conical flask. The volume of red cells (~ 25 cc) was calculated from the sample mass and hematocrit assuming $\rho_c = 1.04$ g/cc and $\rho_s = 1.004$ g/cc and the volume of lysis medium corresponding to $5 V_c$ was computed. Standard saline was added in sufficient quantity to give a final salt concentration of 0.01 M when the appropriate volume of distilled water had been added. Distilled water (~ 100 ml) was then added at ~ 25 cc/min to the cell suspension at room temperature with continuous magnetic stirring. Hemolysis was evident after addition of 20-30 cc H_2O . After the water addition, the suspension was stirred for 20 minutes, then an aliquot of 1.5 M NaCl (14 cc) was added to bring the final salt concentration back to 0.15 M.

In the second procedure, cells were washed in PBS prior to lysis with distilled water. Cell volume computations were made as described above and only distilled water was added to give $V_1 = 14 V_c$. Prior to water addition, the hematocrit was 93-94% and total packed red cell volume was ~ 95 cc. Distilled water was added dropwise from a separatory funnel at a rate of 50 cc/min in one experiment and 20 cc/min in another. With continuous magnetic stirring, hemolysis became evident when about 10% of the water had been added. After addition of the water, sufficient 1.5 M NaCl was added to give a final salt concentration of 0.15 M.

Subsequent to lysis, the treatments were the same for both procedures. The ghost suspensions were incubated for 30 min at $37^\circ C$ to facilitate resealing. Suspensions at $37^\circ C$ were centrifuged 15 min at $\sim 20,000 \times g$ to sediment the red cell ghosts which were washed three times in ~ 20 volumes of standard saline. The washed ghosts were fixed in formaldehyde (4.2.4) or were modified with neuraminidase (4.2.5) and then fixed in formaldehyde.

4.2.7 Lipid extraction

Lipids were removed from fixed red cells by chloroform methanol extraction. The cells were recovered from the aldehyde fixative by centrifugation and were washed twice in > 10 volumes of 0.15 M NaCl and three times in > 10 volumes distilled water. The bulk of water was removed from the cells by three washes in > 20 volumes of absolute methanol and then the cells were extracted by three to four washes in chloroform: methanol = 2:1 by volume. The chloroform methanol washes were intensely colored (red-brown). The chloroform was removed from the system with 2-4 washes in > 10 volumes of absolute methanol. These supernatants were also colored. The cells were then treated in various ways including drying as will be discussed in 4.3.

4.2.8 Miscellaneous materials and methods

Hematocrits were measured in standard microhematocrit glass capillaries following centrifugation for five minutes at 15,000 xg.

Media osmolalities were determined by freezing point depression with an Osmette S automatic osmometer (Precision Systems) calibrated with standard KCl solutions obtained from the manufacturer. Cell volumes were measured electronically with an Electrozone-Celloscope-PDP 8/M Minicomputer package (Particle Data, Inc.) equipped with a 30 or 48 μ m orifice. The instrument was routinely calibrated with standard polystyrene latices of comparable dimensions to the red cells under examination.

Mobility measurements were frequently collected for cells suspended in R-1 buffer, the candidate buffer for the Electrophoretic Separator Experiment scheduled for SPAR flights (20). The preparation and properties have been reported (47) for this buffer which had the following composition:

<u>Substance</u>	<u>Mol. wt.</u>	<u>g/liter</u>	<u>Millimolarity</u>
Na ₂ HPO ₄ ·7 H ₂ O	268.07	0.472	1.76
KH ₂ PO ₄	136.09	0.050	0.367
Na ₂ EDTA·2 H ₂ O	372.24	0.125	0.336

The pH of R-1 is 7.4 at 25°C and the calculated ionic strength is 0.003.

4.3 Results and Discussion

4.3.1 Stability and properties of formaldehyde fixative

The course of heat-induced depolymerization of paraformaldehyde to formaldehyde in PBS and the stability of the fixative following storage at room temperature were examined by point depression measurements. A 1.5% w/v formaldehyde concentration corresponds to 0.50 M. The osmolality of the PBS with formaldehyde is 287 mosmoles/Kg so that if a 100% yield of formaldehyde were obtained from the paraformaldehyde the fixative osmolality should be ~ 800 mosmoles/Kg. Fixative preparations made from different lots of paraformaldehyde gave values ranging from 755 to 780 mosmoles/Kg. The osmolality of the fixative was measured during the heating step (4.2.3) in a water bath at 65°C. Greater than 90% of the final plateau osmolality was obtained by the time the fixative had reached 65°C and plateau values (~ 760 mosmoles/Kg) followed by several minutes. The suspension of paraformaldehyde became clear when the hydrolysis had proceeded to > 95% of the plateau osmolality. The pH and osmolality of the fixative stored sealed in glass at room temperature remained unaltered for at least two months so that polymerization does not occur to a measurable degree during this time period.

Spectral scans of the 1.5% fixative in the 340 to 200 nm region demonstrated only a single absorption peak at 200 nm.

4.3.2 Effects of treatments on electrokinetic properties of red cells

For purposes of screening the influence of modification procedures on the mobilities of red cells, mobilities were measured in simple saline media at two ionic strengths, 0.15 and 0.015. Previous experience had indicated that formaldehyde fixation for undetermined reasons would diminish the mobilities of human red cells at low ionic strength but at physiological ionic strength no significant change could be detected (40). Where native red cells were examined in 0.015 M NaCl, sorbitol was included at 4.7% w/v in order to maintain isotonic conditions, based on the behavior of human red cells. In comparing mobility data collected for native cells in the presence of sorbitol with that for fixed cells in the absence of sorbitol, only a viscosity correction is necessary to account for the influence of sorbitol (8).

Survey data on the effects of various modifications on the mobilities of human, rabbit and goat red cells are given in Tables 5 and 6. In Table 5, all human cell preparations had been treated with 1.5% formaldehyde prior to the collection of the listed mobility measurements. For the native human red cell the mean mobility is 1.08 and 2.40 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ in 0.15 M NaCl and 0.015 M NaCl-4.7% sorbitol at 25°C, respectively at pH 7.2 ± 0.2 . Correction for the 14% elevation by sorbitol in viscosity gives a calculated mobility of 2.74 for human red cells in 0.015 M NaCl without sorbitol. This value can be compared with those for fixed cells in 0.015 M NaCl in order to assess whether the low ionic strength behavior of the native red cell has been altered. For aldehyde fixation only, the mobilities of three lots of fresh human red cells (Table 5) were unaltered at high ionic strength but varied at low ionic strength. The low ionic strength mobilities of subsequent cell preparations have varied within this region from 2.4-2.8 when examined within one to two months of fixation. Thus, the formaldehyde fixation method produced no significant change in the low ionic strength mobility in some preparations and up to a 10-12% decrease in others. Possible reasons for this behavior will be discussed later (4.3.3).

Lipid extraction of formaldehyde fixed human cells followed by drying from methanol did not alter the mobilities of the cells at high or low ionic strength compared with native human red cells. In the experiment listed, the lipid extracted cells had mobilities at low ionic strength which were closer to the calculated value of 2.74 for native red cells than were those of cells from the same lot which had only been fixed in formaldehyde. As will be discussed later (4.3.6), the dried cells were resuspended with difficulty and the yield of cells resuspended from the dry material was low (~ 10 -20%).

Lysis of the red cells (Table 5) was performed by procedures 1 and 2 (4.2.6) for cell lots 2 and 3, respectively. Lysis of the cells produced ghosts with mobilities in 0.15 M NaCl identical to the parent cell population. However, subsequent fixation in formaldehyde gave slightly elevated mobilities at high and low ionic strength in comparison to the results for unlysed fixed cells. This behavior was also observed for 5 week old human red cells. At present, the basis for these mobility increases is not understood. They were consistently observed in 0.15 M NaCl, 0.015 M NaCl as well as in R-1 buffer which has an estimated ionic strength of 0.003.

TABLE 5. EFFECTS OF VARIOUS TREATMENTS
ON THE ELECTROKINETIC PROPERTIES OF HUMAN RED CELLS

Treatment ^a	Cell Lot ^b	Electrophoretic Mobility ^c	
		0.15 M NaCl	0.015 M NaCl
HCHO	1	1.09 ± 0.05 (280)	2.47 ± 0.15 (40)
	2	1.09 ± 0.05 (180)	2.63 ± 0.10 (30)
	3	1.08 ± 0.04 (30)	2.75 ± 0.13 (30)
HCHO, Lip. Ext.	1	1.07 ± 0.06 (30)	2.81 ± 0.14 (30)
Lysis, HCHO	2	1.22 ± 0.06 (20)	2.79 ± 0.12 (20)
	3	1.18 ± 0.05 (30)	2.96 ± 0.11 (30)
HCHO, VCN	1	0.33 ± 0.05 (30)	1.03 ± 0.11 (30)
	3	0.35 ± 0.05 (30)	1.05 ± 0.09 (30)
HCHO, VCN, HCHO	1	0.35 ± 0.06 (30)	1.03 ± 0.09 (30)
	3	0.38 ± 0.05 (30)	1.12 ± 0.09 (30)

^a Treatments are indicated in the order of application to each cell sample: HCHO = fixation of washed red cells in 1.5% w/v formaldehyde in isotonic phosphate buffered saline at pH 7.3; Lysis = osmotic lysis of washed red cells by exposure to hypotonic saline, pH 7.2 ± 0.2 to give 80-95% reduction of cell hemoglobin content; Lip. Ext. = lipid extraction of cells with absolute methanol and chloroform-methanol followed by drying; and VCN = incubation of cells with *Vibrio cholera* neuraminidase in order to remove all of the cell surface sialic acid.

^b Each cell lot number represents a different batch of fresh, washed human red cells which was divided and treated as indicated.

^c Anodic electrophoretic mobility means and sample standard deviations are given for the number of measurements in parentheses. All mobilities were measured at 25°C and at pH of 7.2 ± 0.2 maintained by addition of NaHCO₃ to the indicated NaCl solutions.

The major treatment employed for reducing the mobility of human cells was release of sialic acid by Vibrio cholerae neuraminidase (VCN). The carboxyl group of sialic acid is the major source of negative charge on the human red cell (8,16) and has been indicated to account for up to 80% of the cell surface charge (8). Fixation of cells with formaldehyde is thought to block primarily basic amino groups (18) and has little influence on the susceptibility of sialic acid residues to hydrolytic cleavage by neuraminidase (36). Consequently, cells may be treated with VCN before or after aldehyde fixation without significant changes in the amount of sialic acid which is releasable by the enzyme. There are, however, differences in the final mobilities of unfixed, VCN-treated human red cells depending on the amount of enzyme employed (36). Accordingly, cells with mobilities ranging from ~ 0.35 to zero in 0.15 M NaCl have been observed. This effect which has been attributed to the appearance of positively charged groups on the modified cell surface (36) is misleading with regard to the final mobility of the enzyme-treated cell following fixation. Even though a zero mobility is observed for the unfixed desialylated cell, its mobility increases to about 35% of the native cell mobility following fixation (36).

Treatment of formaldehyde-fixed human red cells (Table 5) with VCN reduces their mobilities by 31-38% in 0.15 M NaCl and 0.015 M NaCl. These values agree well with those observed for cells first treated with the enzyme and then fixed in formaldehyde. When the enzyme-treated fixed red cells are retreated in formaldehyde their mobilities are not significantly altered.

Trypsinization of human red cells was not studied since it has been established that trypsin reduces their mobilities by only about 30% (16). This was not deemed to be a large enough decrease to be of practical value.

Table 6 summarizes the effects of aldehyde fixation and enzyme treatments (trypsin and VCN) on the mobilities of rabbit and goat red blood cells. These cells differ markedly from human red cells in volume (4.3.4) and rabbit red cells have the lowest reported mobilities for mammalian erythrocytes (8). The mobilities of American Dutch rabbit red cells prior to fixation were 38 and 47%, respectively, of the values for human red cells in the high and low ionic strength media (Table 6).

Goat cells (Table 6) were more comparable to human red cells with mobilities of 94 and 79% of the corresponding human cell values. There was, however, some variation in the goat red cell mobilities for samples obtained from two different animals. The values in Table 6 refer to the animal whose cells were employed in the listed modifications. A second animal's red cells gave mobilities of 1.06 ± 0.06 and 2.15 ± 0.08 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ in 0.15 M NaCl and 0.015 M NaCl-4.7% sorbitol, respectively. It is not certain whether these variations represent stable individual differences or differences in the susceptibility of the cells to trauma in the media employed. During washing of the goat red cells for fixation, hemolysis was observed for the cells in 0.15 M NaCl pH 7.4 but not in the PBS employed as the last wash prior to fixation. This behavior prompted tests of the standard saline concentration which was satisfactory and of the solute concentration in the goat plasma which contained added EDTA as anticoagulant. The osmolality of the goat plasma was 381 mosmoles/Kg which is about 25% greater than the value

TABLE 6. EFFECTS OF VARIOUS TREATMENTS ON THE ELECTROPHORETIC PROPERTIES OF RABBIT AND GOAT RED CELLS

Species	Treatment ^a	Electrophoretic Mobility ^b				
		Prefix			Postfix	
		0.15 M NaCl	0.015 M NaCl/Sorb.	u_{sal}^c	0.15 M NaCl	0.015 M NaCl
Rabbit	None	0.41±0.11(60)	1.12±0.10(40)	1.28	-	-
	VCN	Very slow-ND ^d	0.90±0.14(20)	1.03	-	0.97±0.10(20)
	VCN Control	0.42±0.07(10)	0.99±0.09(20)	1.13	-	1.16±0.06(20)
	Trypsin	Very slow-ND ^d	0.55±0.09(20)	0.63	-	1.14±0.12(30)
	Trypsin Control	0.37±0.05(10)	1.12±0.07(20)	1.28	-	1.28±0.10(40)
Goat	None	1.02±0.05(30)	1.89±0.08(30)	2.16	1.14±0.06(20)	2.07±0.10(20)
	VCN	~ 0	≤ 0.2	<0.3	0.47±0.06(20)	0.95±0.11(20)
	VCN Control	-	-	-	1.16±0.08(20)	2.10±0.14(20)
	Trypsin	0.96±0.06(20)	1.86±0.12(20)	2.12	1.18±0.11(20)	2.29±0.15(20)
	Trypsin Control	1.04±0.09(20)	1.79±0.09(20)	2.04	1.14±0.08(20)	2.15±0.12(20)

- a. Treatments indicate those other than fixation in formaldehyde: VCN = *Vibrio cholerae* neuraminidase digestion. VCN controls were incubated with the enzyme buffer containing no enzyme for the same period of time as the VCN samples.
- b. Mean mobilities in $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ $\pm$ standard deviation for the number of measurements in parenthesis. All measurements made at 25.0°C and pH 7.2 $\pm$ 0.2.
- c. Calculated mean mobility in 0.015 M NaCl corrected for the viscosity contribution of sorbitol.
- d. Very slow-ND = Not determined.

of ~ 300 mosmoles/Kg observed for human plasma. Only about 10-15 mosmoles/Kg could be attributed to the added EDTA. In subsequent experiments where 0.21 M NaCl pH 7.4 was used to wash the goat cells no hemolysis was observed. This susceptibility to osmotic lysis may have introduced variability into the electrophoretic data where hemolysis frees hemoglobin and other cell constituents which under appropriate conditions adsorb to the membranes of intact cells and modify their electrokinetic properties. The evidence for human red cells indicates that hemoglobin adsorption is favored by low ionic strength and pH's < 6.9 , the isoelectric point of human hemoglobin (8). At pH's of ~ 7.4 and at ionic strengths of ~ 0.015 hemoglobin adsorption does not present problems. During the collection of the mobility data for goat cells their exposure to low ionic strengths was maintained for less than thirty minutes and the media pH's were 7.3 so that hemoglobin adsorption probably was not a factor if the system behaves similarly to that for human red cells.

Treatment of rabbit and goat red cells in formaldehyde did not appreciably alter their mobilities at low ionic strength. At high ionic strength (0.15), the mobilities of the goat red cells were elevated by $\sim 10\%$.

Incubation controls were included for treatments of goat and rabbit red cells with trypsin and neuraminidase. In the trypsin control samples, the cells were incubated in 0.15 M NaCl containing 0.005 M CaCl_2 . In the VCN control sample, an aliquot of enzyme buffer (0.05 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl and 0.01 M CaCl_2) was added to the cell suspension in the same proportion as used in the enzyme treated samples. These control incubations following fixation had no pronounced effect on the cell mobilities.

VCN produced small decreases in the mobilities of rabbit cells which persisted after aldehyde fixation. In contrast, goat cells behaved like human cells in that VCN decreased their mobilities to about zero prior to fixation but following formaldehyde treatment their mobilities increase to 40-45% of their original values. The mobilities of VCN treated and fixed goat or rabbit cells were slightly lower than those for desialylated human red cells (Table 5). Trypsin substantially decreased the mobilities of rabbit cells but following fixation the cells mobilities increased to nearly control values. Goat red cells were affected little by treatment with trypsin. Slight increases in mobility followed fixation.

The mobilities of formaldehyde-treated human cells were examined as a function of pH and were found to conform to the behavior previously reported (22,24).

An ideal property of any candidate standard cell preparation is a monodisperse mobility distribution. Native human red cells have a very narrow mobility distribution such that it is difficult with standard analytical particle electrophoretic equipment to distinguish between random measurement errors and real mobility dispersity in the population. For the analytical electrophoresis equipment employed in this study, the coefficients of variation were usually about 4-5% for fresh and formaldehyde fixed human red cells at

both ionic strengths (Table 5). Higher values of up to 15% were observed for VCN treated cells in 0.15 M NaCl but were not obtained when the cells were examined at lower ionic strength where values of 10% were obtained. Much of this variation is due to the problems inherent to the measurement of small mobilities where errors in focusing on cells at the stationary level, transient fluid instabilities, etc. assume a greater importance (44). That these factors may predominate over real population dispersity is suggested by the approximately constant absolute values of the standard deviation at each different ionic strength (Tables 5 and 6) regardless of the mobility of the particle population in question.

The use of neuraminidase afforded the possibility of decreasing the mobilities of red cells under mild treatment conditions to a well defined endpoint. A plateau mobility response in the treatment guards against the introduction of mobility dispersity as could be produced with modifications where the mobility distribution of the treated cells is sensitive to the duration of treatment and other factors such as maintenance of homogeneous reaction conditions in the suspension. The data collected to date indicate that neuraminidase treatment is useful for substantially reducing the mobility of a number of types of red cells such as human and goat which contain relatively large quantities of cell surface sialic acid but is not of any advantage with rabbit cells which contain very little releasable sialic acid (48). However, even for human and goat red cells it has not been possible to obtain by neuraminidase treatment fixed cells with less than about 40% of the mobility of the untreated cells.

4.3.3 Storage stability of formaldehyde fixed red cells

Mobility data were collected in 0.15 M NaCl and 0.015 M NaCl at pH 7.2 ± 0.2 for cell preparations stored in fixative for up to ~ 10 months at room temperature. The data are shown in Figure 6 for four different human cell preparations comprised of two from freshly drawn blood anticoagulated with EDTA, one from 3 day old blood bank blood and one from 5 week old blood bank blood. The most striking feature of the curves is the different behaviors observed at high and low ionic strength. At high ionic strength no alteration in mobility is apparent during a ten month period. However, at low ionic strength after about two months the mobilities decay to about 80% of their original values. The decay was most rapid and pronounced for cells prepared from five week old banked blood. The decay in mobility was also observed for fixed human red cells stored in fixative which were examined in R-1 buffer (49). And, smaller mobility decrements of $\sim 10\%$ were noted for stored VCN-treated human red cells when examined in R-1 buffer (49).

Lipid extraction did not prevent the mobility decay. Mobility data were collected for formaldehyde fixed human red cells from 5 week old banked blood. Three cell preparations from the same lot of red cells were tested: a. cells stored in fixative for 8 months; b. cells extracted after 2 months storage with chloroform-methanol to remove lipid and then stored for an additional 6 months in fixative; and c. cells extracted as in b., dried from methanol, and stored dry for 6 months. Mobilities were measured in R-1 buffer at 25°C. The observed mean mobilities were 2.8 $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ for sample a, 2.5 for sample b and 3.5 for sample c.

While the lipid extraction did not prevent the decrease in mobility upon storage, the mobility of the dried preparation was characteristic of freshly fixed human red cells in R-1 buffer which have mobilities of $\sim 3.5 \mu\text{m}/\text{sec}/\text{volt}/\text{cm}$.

The divergent behaviors of stored cells in media of differing ionic strengths (Figure 6) has not yet been satisfactorily explained. As the ionic strength of the medium is decreased, the electrostatic field of charged groups located deeper in the cell membrane increasingly influences the measured mobility due to an increase in the thickness of the electrical double layer (8). Thus, the decay of mobility at low ionic strength may reflect slow changes in the deeper membrane structures. Such changes could include, for example, slow hydrolysis of phospholipids carrying both positive and negative charge groups to form positively charged lipids. However, the observed decay in the mobility of extracted cells would not support this particular mechanism.

A slow deterioration of the electrical resistance of the cell may be involved. The mobilities of osmium tetroxide or potassium permanganate fixed rat red cells (26) and osmium tetroxide fixed sheep red cells (27) are lower by $\sim 30\%$ than the native cells at ionic strengths of ~ 0.01 . At ionic strengths of 0.1 to 0.15, the mobilities of these fixed cells are identical to those of the unfixed red cells. Carstensen (27) has attributed the low ionic strength behavior of the osmium tetroxide fixed sheep red cells to increases in the conductivity of the cell membrane induced by the fixative.

The purity of the paraformaldehyde preparation employed in the formulation of the fixative is now being questioned. In the early electrophoretic studies of aldehyde stabilized red cells (22) the formaldehyde fixative consisted of 0.145 M NaCl into which formaldehyde gas was passed. The gas was generated from paraformaldehyde by heating at 206°C . In subsequent studies of the effects of aldehydes on red cell properties (23) paraformaldehyde was hydrolysed in phosphate buffered saline by heating at $\sim 70^\circ\text{C}$ in order to generate formaldehyde. The latter procedure was employed throughout the ASTP MA-011 Electrophoresis Technology Experiment (1,2,12) and the studies in this contract. During the ASTP studies (2) human red cells fixed in formaldehyde consistently displayed mobilities which were $\sim 20\%$ lower in A-1 buffer than were those of unfixed cells. Time dependent changes in mobility were not established at that time. In the more recent studies covered by this contract, the mobilities at low ionic strength of cells stored in fixative for two months or less (the period of stability) have often been up to 10-15% lower than for native cells. This behavior was not observed by G. Seaman during his early work in which formaldehyde gas was generated to produce the fixative media (32). Recently, the effect of this procedure on the properties of the fixed cells was tested. Formaldehyde was generated by a. heating of paraformaldehyde to 206°C and collection of the liberated gas in 0.15 M NaCl or the phosphate buffered saline, PBS (4.2.3), which has been routinely employed during this contract work; and b. heating of paraformaldehyde in 0.15 M NaCl or PBS to $60-70^\circ\text{C}$. Aliquots of freshly washed human red cells were fixed in each of the four fixatives which contained from 1.41 to 1.74% w/v formaldehyde (see Table 7). The pH of the cell suspensions in 0.15 M NaCl were periodically adjusted to

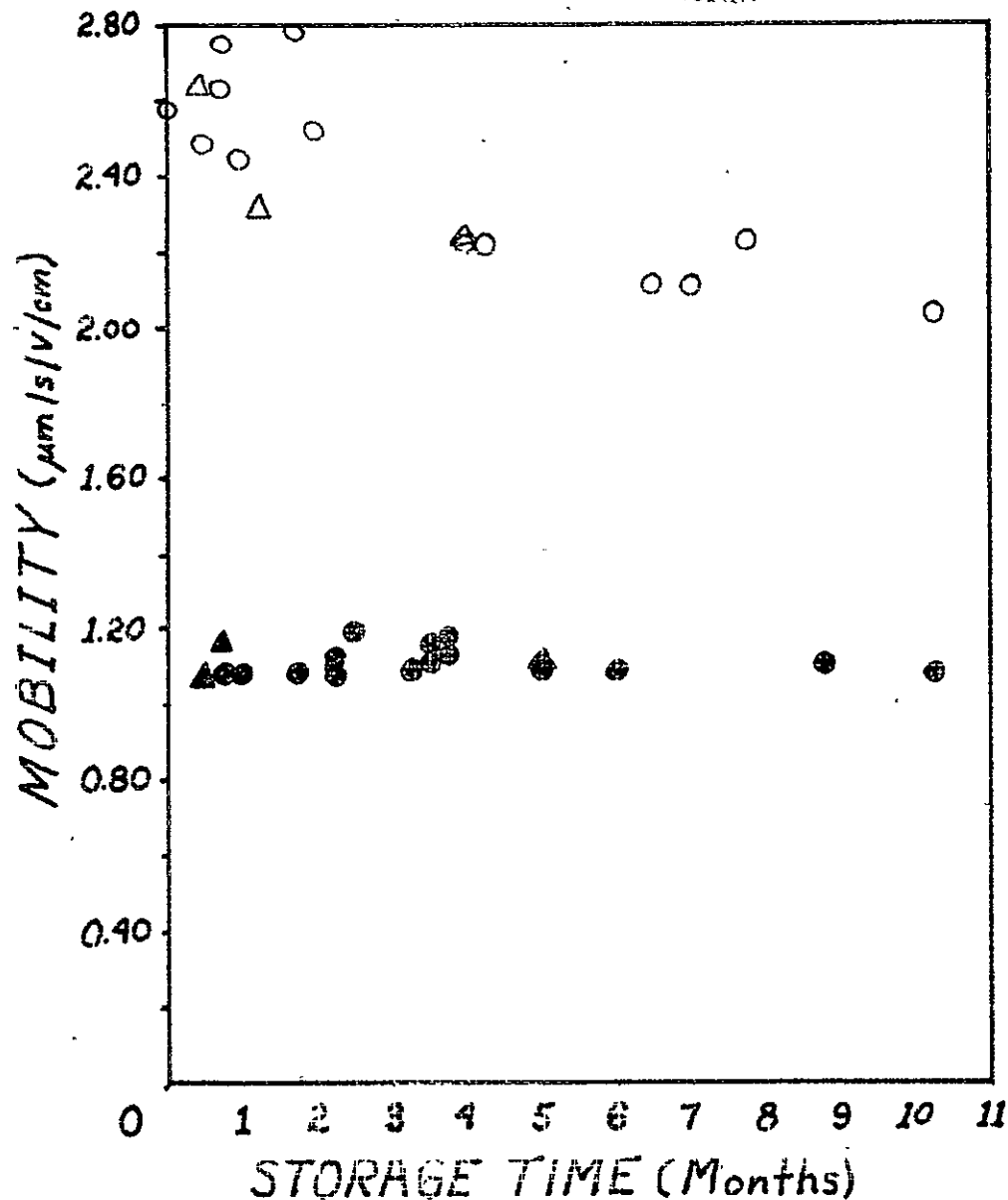


Figure 6. Electrokinetic Stability of Human Red Cells Stored in 1.5% Formaldehyde Fixative. Mean mobilities are plotted for cells in 0.015 M NaCl pH 7.2 ± 0.2 (open symbols) and in 0.15 M NaCl pH 7.2 ± 0.2 (closed symbols) following various periods of storage in the fixative prepared as detailed in section 4.2.3. Cells which were less than five days old at the time of fixation are indicated by circles and cells which were five weeks old when fixed by triangles. Note that based on the mobilities of fresh human red cells at these ionic strengths, the mean mobilities for the fixed cells should be 1.08 and 2.74 at the high and low ionic strengths, respectively, if no changes in electrokinetic behavior have resulted from fixation or storage.

TABLE 7. INFLUENCE OF FORMALDEHYDE PREPARATION METHOD ON THE ELECTROPHORETIC MOBILITIES OF HUMAN RED CELLS AT LOW IONIC STRENGTH

Treatment Medium ^a	Electrophoretic Mobility ^b
HCHO gas/0.15 M NaCl	2.75 ± 0.10 (20)
HCHO gas/PBS	2.73 ± 0.13 (20)
Paraformaldehyde/0.15 M NaCl	2.37 ± 0.18 (30)
Paraformaldehyde/PBS	2.58 ± 0.13 (30)

- a. The formaldehyde concentrations as measured by the titration method in 4.2.3 were 1.74% w/v for those fixatives prepared with formaldehyde gas, 1.47% for paraformaldehyde dissolved in PBS and 1.41% for paraformaldehyde dissolved in 0.15 M NaCl.
- b. Mean mobility in $\mu\text{m}/\text{sec}/\text{volt}/\text{cm}$ ± standard deviation for the number of measurements in parenthesis. The suspending medium was 0.015 M NaCl pH 7.2 ± 0.2 and measurements were collected at 25°C.

~ pH 7 with 1 M NaHCO_3 during the first 24 hours since this medium has no buffering capacity. Mobility measurements were collected after the cells had been in the fixatives for four days (Table 7). The mobilities of the cells from fixatives which had been prepared with formaldehyde gas were essentially the same as predicted from measurements on fresh human red cells at the same ionic strength but with added sorbitol (4.7% w/v). Cells prepared in the fixatives which were made by dissolving paraformaldehyde (procedure b) had lower mobilities which were typical for other human cells prepared by this procedure (Table 5). The lowest mobility was obtained for cells treated in paraformaldehyde dissolved in 0.15 M NaCl. This may be attributable in part to the problem of dissolving the paraformaldehyde in this medium. In order to get complete dissolution of the paraformaldehyde during the standard heating step, 2-3 drops of 0.15 M NaOH were added to 500 cc of the fixative. However, following cooling, the pH was 6.3 and the assayed formaldehyde concentration was lower than for the corresponding fixative made with PBS which suggested a low level of formaldehyde destruction.

Cell hemolysis during fixation was negligible in the fixatives made with formaldehyde gas whereas low levels of hemolysis could be observed for cells in the paraformaldehyde fixatives.

The slightly different aldehyde concentrations for the fixatives was not a critical factor in determining the mobility of the fixed cells. Other experiments established that no significant differences in mobility resulted for cells fixed in 1.5 to 2.5% w/v formaldehyde. The results in Table 7 also indicate that the use of 0.15 M NaCl with pH adjustment or PBS without pH adjustment was not a critical factor. Tests were conducted, however, to determine whether replacing the fixative with fresh fixative after 24 hours affected the mobilities of the cells treated in the two fixatives prepared from formaldehyde gas (Table 7). The mobilities of cells so treated in $\text{HCHO}/0.15 \text{ M NaCl}$ and HCHO/PBS were after 4 days of fixation 2.79 ± 0.10 and 2.80 ± 0.14 , respectively, for the same measurement conditions as in Table 7. These values are slightly higher than those for the same cell preparations which were stored four days without a change of fixative (Table 7) and suggest changing the fixative as a precautionary measure.

At present, the evidence suggests that nonvolatile components of the paraformaldehyde which are eliminated in the formaldehyde gas generation procedure are responsible for depressed red cell mobility at low ionic strength. Chemical analyses are in progress in an attempt to define the nature of those components and will be reported later as part of contract NAS8-32609 (20). Future mobility measurements on these preparations will establish whether these unknown materials are also responsible for the decay in the mobilities at low ionic strength for formaldehyde fixed red cells following storage in the fixative for 3-4 months.

In lieu of these recent studies, the formaldehyde fixative preparation procedure in 4.3.2 should be modified. The fixative should be prepared in PBS but by the dissolution of formaldehyde gas generated by heating paraformaldehyde to 206°C . This may be accomplished by collecting a sufficient quantity of the formaldehyde gas in distilled water to give an estimated

5-10% w/v concentration. The concentration may then be measured by titration as noted in 4.2.3. For one liter of 1.5% w/v formaldehyde fixative, 500 mls of PBS would be mixed with a volume of HCHO in water which contains 15 g HCHO and the volume would be brought to 1 liter with distilled water. It is expected that the storage properties of this fixative will be as described in 4.3.1 for the fixative prepared from paraformaldehyde.

The electrokinetic stability was also tested for human and goat red cells fixed in the paraformaldehyde fixative (4.2.3) and stored in R-1 buffer at room temperature. The mobilities were unaltered for 7-10 days indicating that longer periods of storage are feasible at 4°C in low ionic strength media. One factor was found to seriously affect the electrokinetic stability of the fixed cells in low ionic strength media. Transfer of the cells from the fixative into low ionic strength media before the cells are completely fixed reduced their stability. The mobilities of incompletely fixed human cells in R-1 buffer decayed by ~ 15% in a five day period. This mobility change was accompanied by a visible release of red-brown colored material into the cell supernatant when the cell concentration was ~ 10% by volume. Consequently, a safe fixation period of about 4-6 days should be allowed prior to any storage of the cells in simple saline media.

4.3.4 Characteristics of formaldehyde-fixed lysed human red cells

Three lysed cell preparations were studied until their use as test particles in the electrophoretic separator (20) was precluded by their low optical absorption at 254 nm. This separation device which is scheduled for flight during the SPAR program employed an ultraviolet monitor of the effluent sample stream. Subsequent to this decision, work was suspended on lysed red cells.

As noted in Table 5, the lysis procedure followed by fixation provided cells with slightly elevated mobilities at all ionic strengths tested. No evidence of electrophoretic dispersity resulting from the lysis procedure was obtained. The major type of dispersity encountered was in the final hemoglobin concentrations of the cells and in cell volume. The first lysis procedure (4.2.6) yielded cell ghosts with biconcave discoid shapes typical of the native red cells. Microscopically, heterogeneity in hemoglobin content was apparent. Some cells contained no detectable hemoglobin while others had the appearance of unlysed cells. Measurements of cell density by the phthalate ester method (50) indicated that after fixation, all of the cells had densities less than 1.06 g/cc which was the lowest density ester preparation on hand. The median density of fixed unlysed human red cells was 1.10 g/cc. Consequently, all of the cells were lysed by the procedure but hemoglobin was not equally distributed. This resulted in a range of cell densities which was easily observed in the pack of lysed cells following centrifugation at ~ 15,000 xg for 15 minutes.

Simple high speed centrifugation of the fixed lysed cells was successfully employed in order to obtain cell fractions which were more homogeneous with regard to cell density and hemoglobin content.

Two lysed cell preparations were produced by the second lysis procedure which improved the uniformity of hemoglobin distribution. A sample of the fixed lysed cells in distilled water was fractionated by centrifugation at 27,000 xg for one hour. Approximately 30% fractions were collected which provided separation of the major color intensity zones which could be seen in the centrifuged cell pack. The electrophoretic mobilities of cells from each fraction in 0.15 M NaCl, pH 7.2, at 25°C were not significantly different.

The fixed lysed cells which contained about 5 to 10% of their original hemoglobin content could be visualized with standard bright field optics whereas those cells containing no hemoglobin were very difficult to detect without phase contrast optics.

The fixed-lysed cells had mean volumes of about 20 μm^3 based on electronic particle size measurements. Thus, the lysis procedure resulted in a large reduction in particle volume. The volume distribution, however, was approximately twice as broad as observed for cells treated only with formaldehyde.

The sedimentation rate of all the fixed-lysed cell preparations was much smaller than for the fixed cells. In 0.15 M NaCl at 25°C, the sedimentation rate of fixed cells with densities of 1.10 g/cc is 1 $\mu\text{m}/\text{sec}$. The fixed lysed cells sedimented at about a tenth or less of this rate so that the examination time for electrophoretic measurements is substantially increased.

4.3.5 Red cell volume changes during fixation

Fixed red cells of different volumes and electrophoretic mobilities are useful for making particle mixtures where the electrophoretic resolution of the particles is to be monitored by a nonelectrophoretic method. For example, in preparatory electrophoresis, the resolution of small volume-low mobility and large volume-high mobility particles may easily be monitored by electronic particle size analysis.

The mean volumes of different batches of human red cells subjected to the standard fixation procedure ranged from 70 to 80 μm^3 as measured by electronic particle size analysis. The volumes of rabbit and goat cells prepared by the same procedures were ~ 50 and 15 μm^3 , respectively. The volumes of the fixed human cells were consistently lower than for the native cells whose volumes average about 90 μm^3 under isotonic conditions. The final volumes of the cells could be manipulated to a limited extent by reducing or increasing the concentration of NaCl in the PBS (4.2.3). The volumes of rabbit and human cells were monitored in hypo- and hypertonic PBS formaldehyde media. Where the standard isotonic PBS had a NaCl concentration of 0.12 M, the hypotonic PBS had a concentration of 0.080 M and the hypertonic PBS a concentration of 0.375 M. Fixation in hypertonic 1.5% formaldehyde decreased the cell volumes by $\sim 30\%$ while fixation in the hypotonic fixative produced cells with 15-20% larger volumes relative to the volumes of the cells in the standard fixative. However, when the cell volumes were manipulated by these alterations of the fixative tonicity,

the volume distributions were slightly skewed. Rather than risk trauma to the cells during fixation due to osmotic stresses, it is recommended that cells with different volumes from different species be employed and fixed with media which are isotonic for the given cell type.

4.3.6 Dried fixed cell preparations

It was anticipated that if formaldehyde fixed cells could be dried so that they could be easily resuspended, the preparation would be more manageable for shipment to other laboratories and would have an improved storage life. Two batches of cells were carried through the chloroform-methanol extraction procedure (4.2.7) and various drying procedures were tested. As noted in Table 5, the electrophoretic properties of the extracted and dried cells were characteristic of native red cells. The major problem encountered was obtaining a preparation which could be resuspended in aqueous media.

When fixed cells are suspended in fixative, saline, or distilled water and are dried, a solid mass is formed which cannot be dispersed to form a powder. If the cells are extracted, they still cannot be dried from aqueous suspensions. It was found that washing the extracted cells with absolute methanol and evaporation of the methanol yielded a cake which could easily be crushed with a glass rod to produce a fine powder.

Resuspension of the cell powder was only partially successful. The powder could be wetted with methanol followed by addition of saline media. Mechanical agitation provided suspension of only about 5% of the cells as single cells. Numerous aggregates persisted which could be partially disrupted by sonication to improve the yield of suspended single cells. In suspension, many of the cells had altered shapes which were more like crenated fresh cells than like the biconcave fixed cells which were obtained following extraction.

Methods were not developed which improved the yield of suspendable cells from the dry preparation. The advantages of the dry preparation were supported by the improved storage stability noted in 4.3.3. These advantages may be realized if the cells were to be dried in the presence of traces of wetting agents.

4.3.7 Comments on the use of red cells as standard particles

There are as yet no ideal standard particles for electrophoresis whose electrokinetic and other physical properties may be easily controlled during their production and are completely stable in the variety of experimental environments where they might be employed. Polystyrene latices and aldehyde treated red blood cells were used as standard test particles in the Apollo 16 and ASTP MA-011 electrophoresis experiments, respectively. These particle types share many advantages as standard electrophoretic particles such as physical stability in a variety of media and narrow mobility distributions, however each suffers deficiencies. While physical properties of polystyrene latices such as density, refractive index and color may be manipulated more

easily than for red cells, the electrophoretic mobilities of latices are altered much more readily during experiments by trace levels of for example neutral polymeric materials (11) and surfactants. Experience in this laboratory has indicated that rigorous cleaning of the experimental apparatus which contacts the latices is required in order to maintain narrow mobility distributions. These difficulties may be surmounted by the production of latices with hydrophilic surfaces which are less prone to adsorption of contaminants or aggregation in the case of small latices, both of which can alter their mobilities.

In this laboratory, we have pursued the use of stabilized red cells as standard particles since the mobilities of these particles are not so sensitive to contamination. Their mobilities and various physical properties may be manipulated by chemical or enzymatic techniques. The decay in the electrokinetic behavior of the cells at low ionic strength (4.3.3) has caused concern but current evidence indicates that this problem may be prevented by minor changes in the method for preparing the aldehyde fixative - the importance of which has been realized only recently.

Use of red cells with small volumes, as for example, from goats provides a solution to the problem of rather high sedimentation rates of fixed human red cells in certain experiments. The small size of the cell profoundly decreases its sedimentation rate although its density is comparable to that of the human cell. In addition, other red cells such as those from the rabbit have mobilities of ~ one-half those of human or goat red cells and need only to be stabilized with aldehyde to provide a low mobility particle.

The fixed red cell surface contains numerous hydroxyl and carboxyl groups (8) which may be modified in various ways in order to change the electrophoretic properties of the cells. Zero mobility particles may be produced by esterification of aldehyde treated red cells (24) and it is possible in principle to produce positively charged fixed red cells employing carbodiimide activation of the surface carboxyl groups (18,19) followed by reaction with for example ethylenediamine. Thus, numerous possibilities exist for the production of stabilized red cells with varied physical properties and only a few of these have been explored during this contract period.

4.3.8 Summary comments and recommendations

During the second phase of this contract, candidate preparations of standard particles for electrophoresis have been prepared from fresh and stored human red cells as well as from goat and rabbit red cells. Modification procedures have been tested for controlling electrophoretic and physical properties of the cell preparations. Samples of stabilized red cells were provided to other NASA contractors for use in tests of preparative particle electrophoresis devices. The following recommendations are made as a result of these studies:

1. The most suitable source of red cells for stabilization are fresh red cells or cells which have been stored less than one week at 4°C. Cells

from older blood samples become more sensitive to mechanical and osmotic shocks which are encountered during stabilization and modification procedures.

2. Treatment of red cells with formaldehyde stabilizes their structural and electrokinetic properties. Formaldehyde fixatives for this purpose should be prepared from formaldehyde gas generated by the heating of paraformaldehyde rather than by direct dissolution of paraformaldehyde. Current evidence indicates that undefined materials in fixatives prepared by the latter procedure produce cells with abnormally low mobilities at low ionic strength and these materials may be responsible for the decay of mobility at low ionic strength observed after several months of cell storage in the fixative.
3. Treatment of formaldehyde fixed red cells with Vibrio cholerae neuraminidase provides substantial reductions of 50-60% in the mobilities of human and goat red cells and may be used to generate cells with low mobilities.
4. Human, goat and rabbit red cells have markedly different volumes and may be used to formulate test mixtures of different red cells which differ both in electrophoretic mobility as well as in volume. The relative quantities of each cell type may be determined by microscopic or electronic particle size analysis.

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